CONCEPTS FOR THE DETERMINATION OF PROSTAGLANDINS BY TANDEM MASS SPECTROMETRY

By

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To my loving wife, Paula

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An evaluation of the concepts for the trace determination of prostaglandins (PGs) by tandem mass spectrometry (MS/MS) has been achieved. Results from this work demonstrate the importance of the optimization of various parameters in the collisionally-activated dissociation (CAD) process before performing trace analysis. Dramatic differences in the optimum collision gas pressure for selected-reaction monitoring (SRM) with MS/MS for the determination of prostaglandins E_2 and $F_{2\alpha}$ were observed. The differences in fragmentation behavior were examined through the use of fragmentation, collection and overall CAD efficiency studies. This work shows that the CAD efficiency for the derivatized and underivatized carboxylate anions is significantly different for subtle

structural changes in PGs. A possible explanation has been proposed to explain these dramatic differences.

The advantages and limitations of immunoaffinity purification (IA) for sample preparation of PGs in urine have been investigated. Results show that IA purification coupled with a short 3 m GC capillary column utilizing electron-capture negative chemical ionization (EC-NCI) SRM can provide a selective, sensitive and rapid method of analysis for endogenous levels of PGE_2 in urine.

A systematic study was performed demonstrating the relative trade-offs which exist throughout the entire analytical procedure. Eleven different analytical schemes were systematically evaluated for the trade-offs in sensitivity, selectivity, and total time of analysis. These trade-offs are discussed in relation to how they affect the three basic steps (sample preparation, sample introduction and mass spectrometric detection) of PG analysis. This study indicates that the utilization of a more selective sample preparation method (e.g., IA) with MS/MS can reduce the chromatographic separation time required to achieve the necessary selectivity and sensitivity for PG analysis in urine. However, results show that MS/MS is not necessary if IA purification and a longer chromatographic separation (more selective) technique are employed. This systematic study should be applicable in the evaluation of any analytical procedure for analysis of components in a biological sample. In addition, future work is proposed which should further enhance PG analysis by MS/MS.

CHAPTER 1

INTRODUCTION

Arachidonic Acid Metabolites (Prostaglandins)

The enzymatic oxidation of arachidonic acid (AA) leads to a multitude of biochemically important products (1). Among these substances are prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). Collectively, these compounds are referred to as eicosanoids and constitute what is known as the arachidonic acid cascade (Figure 1-1). Many of these oxygen-containing metabolites have interesting and diverse pharmacological properties and significant medicinal potential (2).

Since, the initial description of PGs in 1935, a vast body of knowledge has accumulated on their physiology and chemistry (1,2). Recently, attention has been focused on PGs of certain series as antitumor agents (3). Evidence indicates that PGs such as prostaglandin E_2 (PGE_2), play an important role as local mediators and modulators of renal blood flow and excretory functions (4). It has been suggested that most of the primary PGs found in urine are derived from renal production (5); consequently, urinary levels of PGs have been applied as an index of renal PG activity in numerous studies. Recently, PGE_2 has seen application in the induction of labor, softening of the cervix and prevention and treatment of stress ulcers (1). While much has been learned about PGs

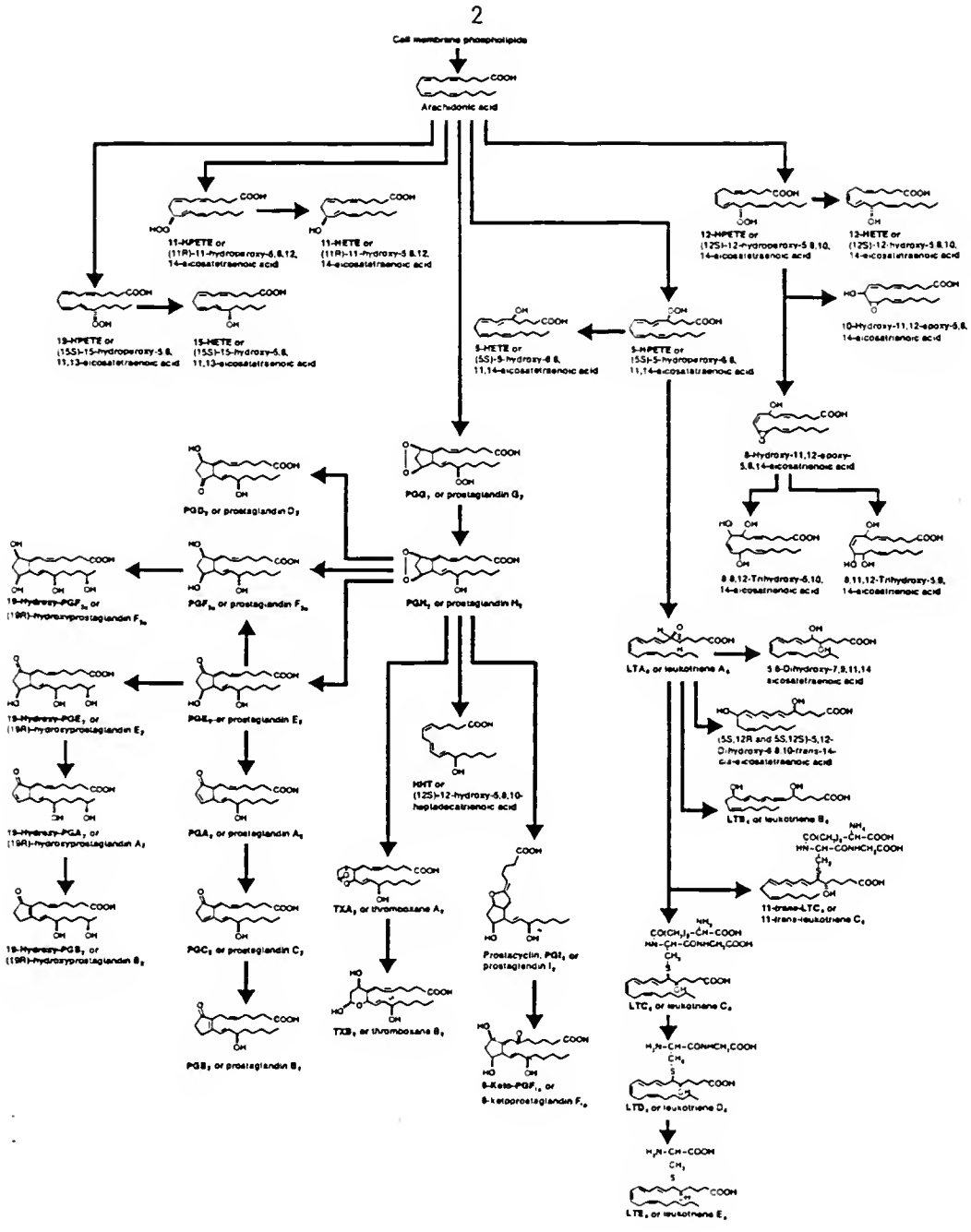


Figure 1-1: Arachidonic acid cascade

biological effects and how they relate to mammalian health, difficulty in measuring low concentrations of PGs in biological systems has hindered the progress of research.

The analysis of PGs and other AA metabolites can be divided into three basic steps; sample preparation, sample introduction and measurement by such techniques as bioassay, radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). It has been shown that with many of the analytical methods frequently used, PG concentrations are often overestimated by as much as a factor of ten (6). This problem can be traced to the lack of selectivity of the entire analytical scheme used for analysis. Therefore, the development of an analytical scheme which provides for accurate, sensitive and selective determination of PGs is needed.

In this chapter, a brief review of the most frequently employed techniques for sample preparation and measurement of PGs will be discussed. More thorough reviews of sample preparation and measurement techniques can be found in the literature (7,8). Recently, a review on AA metabolism with examples of various analysis methods has been published (9). Other, more specific reviews have been written by Traitler (10) and Kelly (11) on mass spectrometric analysis methods of eicosanoids.

Sample Preparation Techniques for Prostaglandins

Sample preparation before the measurement step is extremely important, with the extent of extraction and purification dramatically affecting the validity of the data. In sample preparation of biological

fluids, a traditional technique that is commonly utilized is liquid-liquid solvent extraction (7). This method is time-consuming and usually yields only 80% to 90% recovery of most PGs (12). The degree of clean-up provided by such extractions is limited; further purification is often necessary for body fluids such as urine.

Another popular method for separating PGs from biological matrix components is solid-phase extraction. Three types of solid-phase extraction are commonly employed: (1) amberlite (XAD-2) column; (2) octadecylsilyl (ODS) column (C18); and (3) selective packing materials, such as immunoantibodies. Bradlow (13) described the use of an XAD-2 column that is advantageous when the biological matrix contains a large concentration of proteins. Recoveries using this method have been reported as about 90%. Both solvent extraction and XAD-2 resin extraction procedures are time-consuming and require evaporation of relatively large volumes of organic solvents. Moreover, they are not very selective and give extractions containing extraneous material that must be removed subsequently by various chromatographic purifications. A variety of methods using a C18 column to extract PGs from biological samples have been developed by Powell (8,14) and other researchers (15,16). The solid-phase extraction using a C18 column is rapid, efficient and more selective than solvent and XAD-2 extractions. Recovery with this method has been reported to be greater than 90% in many cases.

Specialized packing materials can provide significantly more selective extraction of specific targeted PGs. A phenylboronic acid column has been used to selectively isolate thromboxane B_2 (TXB_2) and its metabolites (17). The recovery of radio-labeled TXB_2 after extraction was

reported at 90%. Potentially, an even more selective approach to sample preparation is to combine the extraction and purification steps into one procedure. This has been accomplished by using an antibody-mediated extraction procedure developed by Krause et al. (18). Basically, the prostaglandin-antibody was coupled to cyanogen bromide-activated Sepharose 4B and used as a stationary phase for the extraction of PG from the samples. The antibody was coupled to Sepharose and packed into a Pasteur pipette. The plasma samples were then applied to the gel in the column. This one-step extraction-purification method has shown improved specificity and sensitivity. Similar methods have also been employed by Hubbard (19) and Vrbanac (20) for the analysis of TXB_2 and 6-keto-$PGF_{1\alpha}$ in urine. Another approach to exploit the high selectivity of antibody-antigen reactions for sample extraction is double-antibody precipitation. This technique has been used for preparation of plasma samples before HPLC analysis for (15R)-15 methyl-PGE_2 (21).

In summary, the advantages of extraction (either solvent or solid-phase) are: (1) it eliminates some extraneous material, thereby imparting greater specificity to the assay; and (2) it improves sensitivity of the analysis by concentrating the material. The disadvantages are: (1) it is time-consuming; (2) a "carry-over" of non-eluted analyte may occur (if the same column is reused) effecting the validity of subsequent assays: and (3) the extraction efficiency of the procedure is variable. Simple solvent or solid-phase extraction has been shown to yield samples that do not permit accurate validity of PG quantitation. Improved validity of subsequent quantitation has been observed after further purification

steps. A more detailed explanation of solid-phase extraction and immunoaffinity (IA) purification can be found in Chapter 2.

Three types of chromatographic purification are described below: (1) silica acid column chromatography; (2) thin-layer chromatography (TLC); and (3) HPLC. Group separation of PGs and related compounds is conveniently performed by silica acid column chromatography (22,23). Recoveries were reported for this purification method of 85% to 90%. PGs separated by silica acid chromatography usually require further purification by TLC or HPLC prior to quantitation by RIA or GC/MS.

Separation of PGs by TLC was first investigated by Green and Samuelsson (24). TLC is the most commonly used method for separation of PGs because of its efficiency, simplicity and economy compared to other chromatographic procedures. The major groups of PGs (A, B, D, E, F and 6-keto-$PGF_{1\alpha}$) were readily separated on a silica gel plate using various solvent mixtures (25-27). The disadvantages of TLC are its low recovery yields (typically 80%) and the lengthy procedures required for separation of closely related compounds. Prostaglandin-related compounds with similar behavior are often observed to migrate in a similar way even in different solvent systems. Such problems can be avoided by using two-dimensional TLC. A considerable improvement of resolution is achieved by combining two solvent systems with different chromatographic properties. Two-dimensional TLC analysis of PGs and related compounds has been reported from a few laboratories (8,28-30).

The conventional techniques of column chromatography and TLC usually suffer from poor chromatographic resolution and the need to use several solvent systems to adequately separate arachidonate metabolites. HPLC has

been used successfully for the separation and purification of PGs from biological sources since 1976 (31). This technique offers several advantages: (1) there is high resolution of closely related compounds; (2) good reproducibility is possible; and (3) fractions containing PG peaks can be automatically collected and later quantitated by RIA, GC/MS or scintillation counting of radio-labeled metabolites. Both normal-phase HPLC on silica acid (32-34) and reverse-phase HPLC on octadecylsilyl silica (35,36) have been used to separate the cyclooxygenase products of arachidonic acid. However, HPLC can be an extremely lengthy technique for purification and can yield low recoveries on the order of 60%.

Techniques for Determination and Measurement of Prostaglandins

A number of analytical methods have been developed for the detection and measurement of PGs to study their physiological and pharmacological effects. Among those, bioassay, RIA, HPLC and GC/MS are most widely used for the quantitation of PGs in biological fluids.

Bioassay. Biological techniques and bioassay have contributed greatly to the development of techniques for detecting and quantitating AA metabolites (37,38). In general, bioassay has been highly beneficial in establishing the biological significance of the unstable products of AA metabolism. However, it provides only approximate quantitation and relatively low selectivity.

Radioimmunoassay (RIA). RIA of PGs was introduced in 1970 by Levine and Van Vunakis (39) with assays developed for PGE_1 and $PGF_{2\alpha}$. The literature has been expanding rapidly, and a large number of RIAs for PGs, TXs and LTs have been reported. RIA is based on the competition between

radio-labeled and unlabeled molecules of a particular compound for binding sites on an antibody directed against the same compound. The amount of labeled compound is known and constant for all the tubes in an assay, whereas the amount of unlabeled substance is either known and varied (standard tubes) or unknown (sample tubes). A tube with no antibody present is required as a "zero binding" tube. A tube containing no unlabeled substance is also required as a "maximal binding" tube. When larger amounts of unlabeled substance are present, the radioactive molecules are displaced from the binding sites. The radioactivities of the unbound fraction and antibody-bound fractions are usually separated by dextran-coated charcoal or double-antibody methods, and the radioactivity of either or both fractions is determined. The amount of unlabeled compound in a sample tube is then obtained by comparison with the standard tubes.

RIA has certain advantages over other quantitative methods, the most important being its high sensitivity, with detection limits as low as a picogram per sample. The precision and accuracy frequently compare favorably with other methods. RIA is relatively rapid and also has high sample capacity; for example, 100 samples can be analyzed within one or two days, including radioactivity measurements and data processing.

RIA also has some drawbacks. First, the method is not entirely specific under all circumstances. It is difficult to obtain a specific antibody with a minimum of cross-reactivity and high affinity. Biological samples, especially biological fluids (urine or plasma), usually need to be purified through extraction, column chromatography, TLC, or even HPLC before analysis by RIA. Appropriate purification steps are time-

consuming, but frequently necessary to remove most interfering compounds and yield a specific assay with valid results. Other disadvantages of RIA include the potential risk inherent in using radioactive materials and the high cost of using disposable glassware, utensils, counting vials and large volumes of scintillation fluid.

High-performance liquid chromatography (HPLC). HPLC has proven to be useful for purification of PGs after an initial extraction procedure. The HPLC technique is a good qualitative method; however, quantitation is rather limited, especially for PGs. Terragno et al. (36) have found that the highest molar extinction coefficient occurs around 192.5 nm for major PGs, yielding detection limits in the nanogram range. Recently, a more sensitive method using HPLC with a postcolumn derivatization and fluorescence detection has been developed for eicosanoid quantitation. Watkins and Peterson (41) developed a method to measure AA metabolites by reverse-phase HPLC followed by formation of the ester derivative with P-(9-anthroyloxy) phenacyl bromide. The disadvantages of HPLC are that this technique can be lengthy and a relatively large volume of sample is required for adequate detection of low levels of PGs in biological fluids.

Gas chromatography/mass spectrometry (GC/MS). GC/MS is the analytical method of choice for the identification, characterization, and quantitation of the products of the arachidonic acid cascade. Offering both high sensitivity and selectivity, GC/MS has become the "gold standard" for the analysis of PGs. Traditionally, GC/MS was used for strictly qualitative analysis, with studies done on the determination of the structures of several prostaglandins (42). Identification and

characterization of many prostaglandins and their metabolites were performed by electron ionization/mass spectrometry through the early to mid-1960's. In 1967, reports on eicosanoids first appeared, with limits of detection in the low ng/mL range (42,43). The use of selected-ion monitoring (SIM) with positive chemical ionization (PCI) and electron-capture negative chemical ionization (EC-NCI) for quantitation significantly improved the detection limits achieved by GC/MS.

The discussion that follows will focus on components of the analytical technique of GC/MS for the analysis of PGs. In a typical qualitative or quantitative analysis for PGs by GC/MS, the following steps are performed: (1) sample preparation (extraction and purification); (2) derivatization; (3) gas chromatographic separation; (4) ionization; and (5) mass spectrometric detection. In the following pages, these analytical steps will be discussed in reverse order, highlighting the mass spectrometric component of the analysis, rather than sample preparation which was discussed in detail earlier.

In mass spectrometric analysis, the quantitation of trace levels of PGs is commonly performed by utilizing an isotope-labeled analog of the compound of interest, with selective monitoring of the ions of each. Since its introduction in 1967, stable isotope dilution (44) has been the method of choice for quantitation. Many uses of stable isotope labeling with SIM can be found in the prostaglandin literature (45-54). Both high resolution mass spectrometry and low resolution mass spectrometry have been employed for analysis of PGs. High resolution can reveal the elemental composition of ions, which is helpful in identifying new compounds. Low resolution is used for trace analysis despite its lower

selectivity. Examples of both techniques can be found in the literature (55-59). A great deal of research has been devoted to trace analysis of eicosanoids and their metabolites in all types of biological fluids, with most determinations done in plasma and urine (48,49,60-63). The amounts that have been analyzed are from the low ng to low pg/mL range, with limits of detection as low as 50 fg reported in one study (64).

Three types of ionization are used today for most PGs analyses: electron ionization (EI), positive chemical ionization (PCI), and electron-capture negative chemical ionization (EC-NCI). EI/MS, as discussed earlier, is most often used for structural elucidation and identification of new compounds. EI mass spectra give structurally useful fragmentation patterns, although the molecular ion may be weak or even absent. For trace analysis, typical limits of detection with EI are approximately 100 pg/mL (57). PCI and EC-NCI are "gentler" ionization techniques, generally producing less fragmentation, with a more prominent (pseudo-) molecular ion. Thus, these techniques are useful for confirming molecular weight, and for trace analysis by selected-ion monitoring. PCI has been shown to be helpful in characterization of thromboxanes and prostaglandins (65). Limits of detection vary for PCI and EC-NCI, depending on both the compound and the reagent gas selected. Many types of chemical ionization reagent gases have been used, but methane and isobutane are the most common. Most trace analysis studies are now performed with EC-NCI with methane as the reagent gas. Detection limits are generally in the low pg/mL range, although limits as low as 50 fg/mL have been reported (59). The three ionization techniques have been

compared for trace analysis of PGs, including limits of detection and spectra obtained with each ionization technique (49,57).

Gas chromatography is generally used to separate the eicosanoids from each other and from other potential interferents prior to their identification or detection by mass spectrometry. The first GC/MS analyses were accomplished with packed gas chromatography columns, which were used extensively until the development of fused silica capillary chromatographic columns. Until 1982, approximately equal use was made of packed and capillary column techniques, but capillary chromatography has led to better separation of closely related compounds. Coupled with negative chemical ionization, it has allowed researchers to achieve limits of detection in the low pg/mL range. These advantages have provided higher sensitivity and selectivity in eicosanoids analysis. However, packed column chromatography still has a role in prostaglandin analysis. One recent study (66) showed the advantages of packed columns for highly contaminated samples that exceeded the capacity of capillary columns. Researchers have recently recognized the value of introducing the capillary column directly into the ion source of the mass spectrometry (67). This avoids problems with contamination, adsorption, and decomposition of analytes (which can be severe with PGs) on active surfaces in other GC/MS interfaces.

Derivatization of PGs has been important in their analysis, both to increase their volatility for gas chromatography separation and to provide for efficient EC-NCI to increase sensitivity of the GC/MS method. Today, the methylester/methoxime/trimethylsilyl ether of PGs is the most frequently cited derivative in GC/EI/MS analysis (63,64). However, it has

been shown that these derivatives are susceptible to hydrolysis, often producing ions that are not optimal for selective-ion monitoring (68). This is due to the low relative intensity of the high mass ions which are optimal for quantitation.

The derivatization of PGs for GC/EC-NCI/MS seems to be standardizing on the methoxime/trimethylsilyl ether/pentafluorobenzyl ester (MO/TMS/PFB) mixed derivative (50,51,69-72). Derivatization with perfluorinated acid anhydrides has been increasingly used for both qualitative and quantitative work (69). These anhydrides usually incorporate a silylating reagent such as N-(tetra-butyldimethylsilyl)/N-(methyltrifluoroacetamide). This gives hydrolytic stability and increases high mass ion intensity for optimal use of selective-ion monitoring. The use of such derivatives also eliminates detection of many nonprostaglandin carboxylic acids, due to their ability to derivatize with the carbonyl, rather than, or in addition to, the carboxyl group. This makes these derivatives highly attractive for detecting trace quantities of prostaglandins in biological matrices (73).

Recent Analytical Advances

GC/MS remains the workhorse technique of PG research; however, tandem mass spectrometry (MS/MS) and soft ionization techniques such as fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS) and liquid chromatography/mass spectrometry (LC/MS) are being effectively employed. The sensitivity and selectivity of GC/MS/MS compared to GC/MS has been studied in reports (74-76) utilizing both EI and EC-NCI. The advantages of GC/MS/MS have recently been exploited for

the trace analysis of PGs in biological samples (20,77,78). These studies have been performed on both sector and quadrupole instruments. The high selectivity of MS/MS makes it possible to perform analyses with minimal sample preparation. MS/MS also minimizes or eliminates the need for chromatographic separation in many cases, making the analysis extremely rapid. MS/MS experiments have recently been reported in the literature for analysis of underivatized prostaglandins (79,80).

In addition, with improved instrumentation has come the technique of FAB or LSIMS (81-83). This method has aided structural elucidation, as well as characterization of many PGs. LC/MS has become increasingly popular in the analysis of PGs (84,85), as in all areas of chemistry. LC/MS has the ability to analyze polar, thermally labile, and high molecular weight eiconsanoids, and it saves time in sample preparation. LC/MS with thermospray ionization (TSP-LC/MS) has been used by several researchers to detect PGs and TXB_2 at limits of detection as low as 10-300 pg (on column), after derivatization with (diethylamino)ethyl chloride (86). A series of PG standards were analyzed and investigated to show the increase in sensitivity resulting from a post-column derivatization which formed the methyl ester (87). The sensitivity is still not equal to the GC/MS methods commonly employed. This is the limiting factor of LC/MS for the analysis of PGs; however, there is reason to believe that the necessary improvements in sensitivity can eventually be obtained. LC/MS is a good qualitative technique which is still in its infancy. The advantages to be gained in simplified sample preparation and the ability to directly analyze the more polar eiconsanoids will stimulate further improvements.

Another recent MS/MS technique which is promising is ion trap (IT)MS/MS. The ITMS offers the potential for very selective and sensitive GC/MS/MS analysis. In the ion trap, ion formation and mass analysis occur in the same region (tandem-in-time), whereas, in tandem mass spectrometry these two processes occur in different regions (tandem-in-space). The analysis of PGs by this method has been reported by Strife (88,89). This work shows the unique advantages of high sensitivity MS/MS,with nearly 100% conversion efficiencies of parent to daughter ion in MS/MS experiments.

This section of Chapter 1 has shown that much progress has been made in the area of PG sample preparation and quantitation. Many limitations remain, especially when the sample size is limited. In the chapters to follow some of these limitations will be addressed and new analytical schemes will be evaluated.

Strategies for Mixture Analysis by MS/MS

Since the development of tandem mass spectrometry (MS/MS) in the 1970's, it has recently gained rapid acceptance as an exceptional analytical tool for mixture analysis (90-93). MS/MS has the ability to provide rapid, sensitive and selective analysis of complex biological samples, often with minimal sample clean-up (94,95).

The MS/MS scan modes utilized in these studies are depicted in Figure 1-2. In mixture analysis, chemical ionization of a mixture is often utilized in the ion source of the mass spectrometer to produce ions characteristic of the components in the mixture and to achieve a spectrum with few fragments. Separation of the analyte from the matrix components

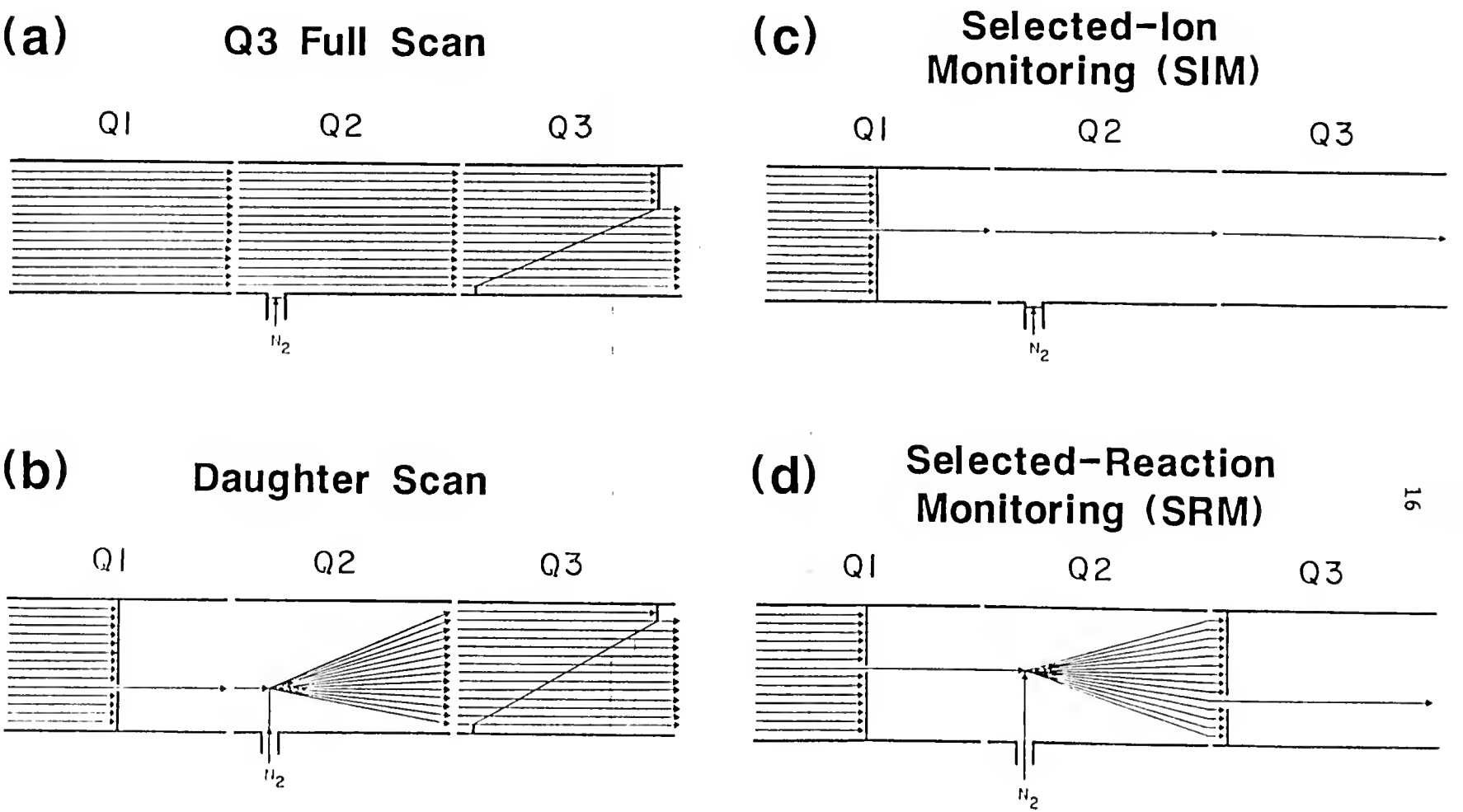


Figure 1-2: Tandem mass spectrometry scan modes

is achieved by the mass selection of a characteristic ion of the analyte by the first mass analyzer (Q1). The selected parent ion undergoes collisionally activated dissociation (CAD) through collisions with neutral gas molecules in the fragmentation region (Q2) to yield various fragment or daughter ions. Subsequent mass analysis of the daughter ions by the second mass analyzer (Q3) results in the analytical signal. This method of MS/MS analysis corresponds to a daughter scan (Figure 1-2b).

Although this operational mode is highly selective, this full-scan daughter mass spectrum usually does not exhibit sufficient sensitivity for trace analysis of an analyte in a complex matrix. Therefore, the scan mode of selected-reaction monitoring (SRM) is commonly employed (Figure 1-2d). A characteristic daughter ion, typically the most abundant, resulting from the fragmentation of the selected parent ion of the analyte, is selected by the second mass analyzer for monitoring. SRM is analogous to the selected-ion monitoring (SIM) (Figure 1-2c) commonly used to obtain maximum sensitivity in conventional GC/MS. Thus, an enhancement in sensitivity is obtained at the expense of a gain in selectivity. In addition to these MS/MS modes, the tandem mass spectrometer can be operated as a normal MS by allowing all ions to pass through one mass analyzer (Q1 or Q3) and the collision cell (Q2), then scan the other mass analyzer (Q3 or Q1) to produce a normal mass spectrum (Figure 2-1a). Optimization of many of these operational modes have been evaluated throughout these studies and will be discussed in further detail as to their significance in the trace determination of PGs.

Important Parameters for Trace Analysis

In order to perform trace analyses successfully, it is necessary to think in terms of the four "S's" of analysis: (1) sensitivity; (2) selectivity; (3) speed or analysis time; and (4) $ or cost. In the determination of pure analytes, sensitivity can be a very useful criterion; however, when required to determine an analyte in a complex matrix, sensitivity alone may become meaningless. This is due to the fact that chemical interferents in the matrix may themselves produce a response or interfere with the signal of the analyte. Therefore, the factor which may determine the smallest amount of analyte which can be determined accurately is the second "S", selectivity. The selectivity can be described as the ability of the method to distinguish the signal of the analyte from that of the chemical interferents (so-called chemical noise). The limit of detection (LOD), which depends upon both the selectivity and sensitivity, is defined as the smallest amount of analyte which can be detected.

The LOD required in trace analyses can be achieved by improving the selectivity of the analytical scheme. Normally, this is accomplished through the use of extensive sample clean-up and separation to enhance the analyte signal with respect to the matrix components signal. These extractions and purifications increase the possibility of sample contamination and sample loss. Additionally, the methods necessary to increase selectivity may become time-consuming and expensive, thus the final two "S's", speed of the analysis and cost effectiveness may not be optimum.

The Four Steps Involved in Trace Mixture Analysis

The analytical scheme for trace determination of an analyte in a biological sample by MS/MS involves four basic steps: (1) sample preparation; (2) sample separation/introduction; (3) ionization; and (4) detection. When developing an accurate, reliable and specific method for mixture analysis, a range of selectivity, sensitivity, time and cost are observed for the four steps.

In sample preparation, a rapid, low cost and selective procedure is desired. This can be achieved through the proper choice of extraction, purification or derivatization methods which satisfy any or all of the four "S's". The second step involves separation of the analyte of interest from any matrix components which have not been eliminated by the sample preparation methods. Typically, in MS/MS, gas chromatography is employed for separation, if the analyte exhibits sufficient volatility. Separation of components can be accomplished on short capillary GC columns (3 m or less), when the sample has been adequately cleaned-up (96). Short GC columns can only be utilized for separation of complex samples if the sample preparation methods have the necessary selectivity. The choice of an ionization method is based on the type of analysis required and the analyte which is to be analyzed. In the low level trace determination of analytes in biological samples, a "soft" ionization method (e.g., chemical ionization) is usually selected which yields an intense molecular ion with few fragments. Furthermore, for analytes which are highly electron-capturing (or can be derivatized), electron-capture negative chemical ionization (EC-NCI) may be chosen in order to achieve the highest sensitivity. Finally, the detection by MS/MS involves the optimization

of the parameters which constitute the operational modes which were discussed above.

Overview of Thesis Organization

This thesis is divided into seven chapters. Chapter 2 describes the sample preparation concepts and methods employed for this work. Results from recovery studies on various types of extraction columns and their characteristics are discussed in detail. The concept of immunoaffinity purification is introduced and investigated.

The third chapter emphasizes the importance of optimizing MS/MS parameters for trace determination of PGs. Optimization studies for selected-ion monitoring (SIM) and selected-reaction monitoring (SRM) for PGE_2 and $PGF_{2\alpha}$ are described and the results discussed.

Chapter 4 presents a study of the differences in the CAD efficiency of two structurally similar PGs (PGE_2 and $PGF_{2\alpha}$). Collision energy and collision gas pressure studies of the carboxylate anions of four PGs are evaluated and hypotheses for the differences noted are put forth.

The results of the quantitation study of endogenous PGE_2 in urine can be found in Chapter 5. The advantages and disadvantages of various analytical schemes are pointed out. These schemes are systematically evaluated for the trade-offs in sensitivity, selectivity and time of analysis. The trade-offs are discussed in relation to how they are affected by the three basic steps (sample preparation, sample introduction and detection) of PG analysis.

Chapter 6 includes an evaluation of the rapid analysis techniques of direct solids probe/MS/MS and direct chemical ionization (DCI)/MS/MS

utilizing an abbreviated derivatization procedure. The advantages and limitations are discussed and the results of a quantitation study of endogenous PGE_2 in urine are presented.

The final chapter reviews the conclusions which were drawn from this work. This chapter points out the potential of GC/MS/MS with selective sample preparation and short GC capillary columns to determine endogenous levels of PGE_2 in urine. The importance of a systematic study of the entire analytical scheme is finalized. In addition, future work is proposed which should further enhance PG analysis by MS/MS.

CHAPTER 2

SAMPLE PREPARATION STUDIES

Introduction

Sample preparation is an important step in any analytical methodology. This step prepares the sample for the detection method and can dramatically affect the validity of the data obtained. The two main parts of sample preparation for gas chromatography/mass spectrometry (GC/MS) are sample purification and derivatization. When considering sample purification, selectivity and speed of the method are of vital importance. A method which is extremely selective can eliminate matrix interferences and reduce the separation needed in GC. Sample throughput is always an important factor in any analytical method. A rapid sample purification step can greatly reduce the total time of analysis.

The other main part of sample preparation is derivatization. Many compounds are not directly amenable to GC. The thermal lability of prostaglandins (PGs) makes it impossible for them to pass through a GC column intact without first undergoing derivatization. This derivatization increases the volatility of the compound and reduces the interaction of the polar substituents on the compound with the stationary phase of the GC column. In addition, derivatization can add sensitivity and/or selectivity for detection of a compound. Many organic derivatization reactions with PGs enhance the efficiency of electron-

capture negative chemical ionization (EC-NCI) mass spectrometry (51, 70-72). Electron-capture NCI with derivatization produces much simpler mass spectra and the major fragment ions occur at the high mass range (50,69). Thus, these two features combined with the higher ionization efficiency of EC-NCI provide added sensitivity and selectivity needed in trace determination of PGs.

Concepts for Solid-Phase Extraction

Solid-phase extraction (SPE) has emerged, in the last ten years, as the method of choice for isolation and purification of arachidonic acid metabolites (8,14-16). SPE has the advantage of using low volumes of solvents and high recoveries of 90% to 100% for most PGs. Rapid extractions are usually possible with simple procedures. This results in a rapid inexpensive extraction technique. The concept of SPE is based on the selective retention of the analyte by a sorbent bed as a solvent in which the analyte is dissolved is passed through the column. This idea is displayed graphically in Figure 2-1. A sample containing analytes (A) and interferences (I & M) is passed through the sorbent. The sorbent selectively retains analytes (A) and some interferences (I). However, at the same time, many interferences (M) pass unretained through the sorbent. Appropriate solvents are then used to wash the sorbent, selectively eluting previously retained interferences (I), while the analytes (A) remain on the sorbent bed. Purified, concentrated analytes (A) are then eluted from the sorbent.

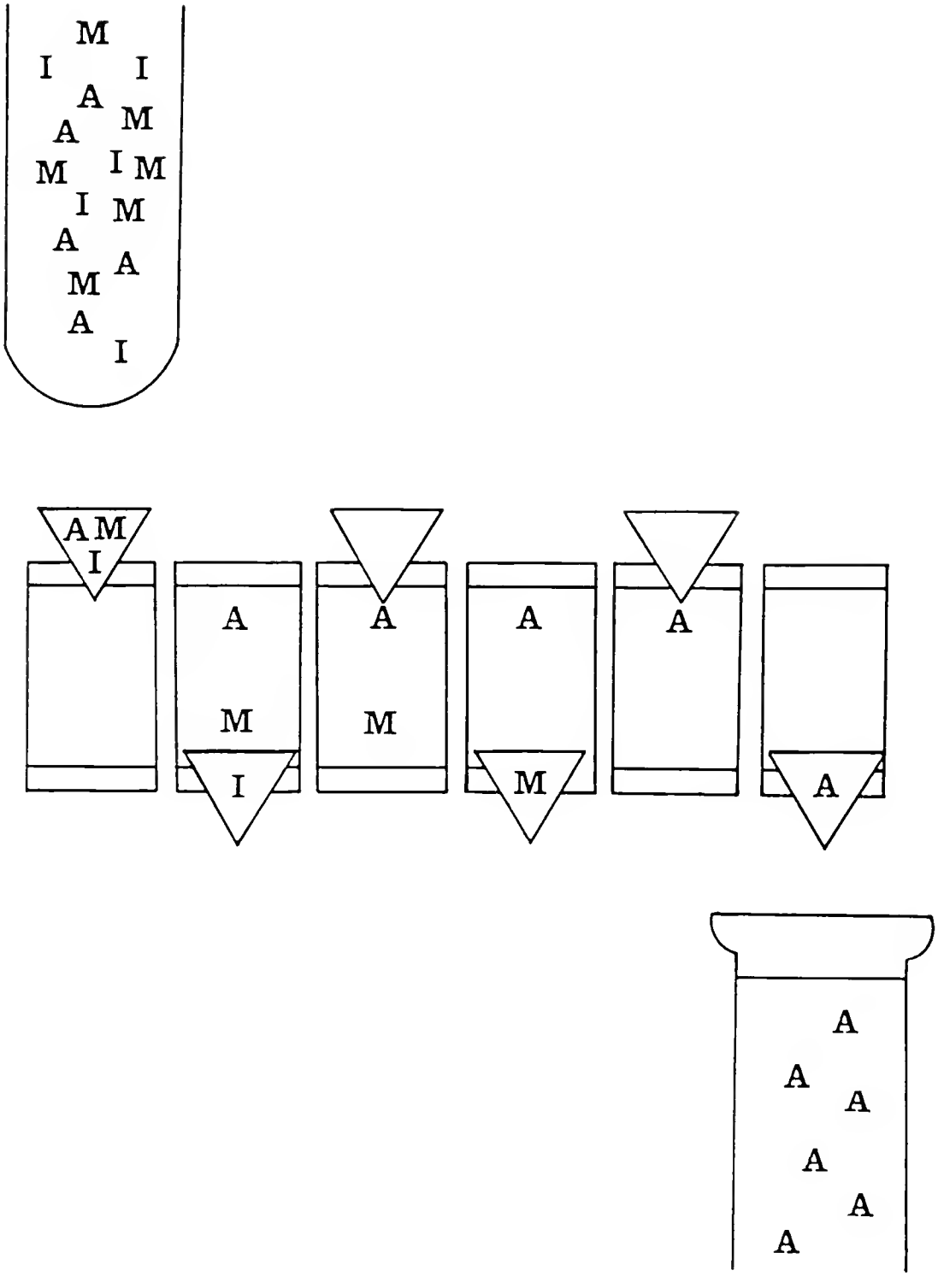


Figure 2-1: Concept of solid-phase extraction:
A = analyte; I & M = interferences.

Sorbent/Analyte Interactions

Three types of chemical interactions are commonly employed in solid-phase extractions (97). The first is the non-polar interaction which occurs between the carbon-hydrogen bonds of the analyte and that of the sorbent. Virtually all organic compounds have some non-polar character, thus these types of interactions are the most commonly used to retain analytes on sorbents. The forces which are involved in such non-polar interactions are "van der Waals" or dispersion forces (97,98). The most widely used sorbent in non-polar interactions is octadecyl silane bonded to a silica substrate which is called C18. Many compounds can be retained by a C18 sorbent, thus it is a very non-selective sorbent. In general, non-polar solid-phase extraction is the least selective extraction procedure. The concept of non-polar interaction is comparable to that of reverse-phase chromatography. Retention of the analyte by non-polar interaction is facilitated by a solvent more polar than that of the non-polar sorbent. Elution is then accomplished by utilizing a solvent with sufficient non-polar character to release the retained analyte from its interaction with the sorbent.

Other interactions which are common for SPE are polar interactions. These interactions are exhibited between many sorbents and functional groups on analytes. All bonded silica exhibits polar interaction due to the polar nature of the silica substrate to which the sorbent is bound (97,98). Polar interactions include hydrogen bonding, dipole/dipole, pi-pi and many more interactions in which the distribution of electrons is unequal in the atoms of the functional groups. This property of polar sorbents allows an analyte which contains a polar functional group to

interact with a polar group on the sorbent. Groups that exhibit these types of interactions include hydroxyls, amines, carbonyls and other groups containing hetero-atoms such as oxygen, nitrogen, sulfur and phosphorous. The most common polar sorbents are silica, diol, aminopropyl and cyanopropyl. Polar sorbents function similarly to the interactions found in normal-phase chromatography. Non-polar solvents are used to promote retention of the analyte on the polar sorbent. Then a solvent, more polar than the sorbent, is utilized to elute the analyte.

The third type of interaction is ionic. This occurs when an analyte carrying a charge (either positive or negative) interacts with a sorbent carrying a charge opposite to that of the analyte. Ionic interactions are more selective than non-polar and polar interactions and can be controlled by adjusting the pH of the sample solution. It is essential to know about the functional groups on the sorbent and the analyte because both of these need to be charged to facilitate ionic interaction. Two classes of ion-exchange interaction exist, cationic (positively charged) and anionic (negatively charged). Examples of cationic interactions include the interaction of amines and certain inorganic cations with carboxymethyl, sulfonylpropyl and benzenesulfonylpropyl sorbents. Anionic interactions occur when sorbents containing primary, secondary, tertiary and quaternary amines interact with carboxylic and sulfonic acids, phosphates and similar groups on an analyte.

Recently, covalent interactions have been exploited for extraction of specific types of compounds (99). Covalent chromatography is highly chemically selective, involving an interaction of greater energy than is employed in the other extraction methods. Retention of the analyte occurs

when a covalent bond can form between it and the sorbent. A change in the solvent environment facilitates elution of the analyte. This is commonly accomplished through the use of solvents with various pH's. One example is phenyl boronic acid (PBA) which has been immobilized for the selective retention of compounds with 1,2- or 1,3-diols such as catecholamines and thromboxanes (17,100).

Many of the sorbents discussed above may exhibit more than one interaction. Both polar and ionic interactions due to the silica substrate used can occur in all sorbents. In the case of the PBA, non-polar, polar and ionic interactions can occur as secondary interactions within the sorbent. The interactions which occur with a particular sorbent are a function of the sample matrix and the solvent used for washes and elution.

Sorbent Selection

The problem encountered in this analysis was to develop a solid-phase extraction to selectively isolate PGs from interferences in urine. Evaluation of different sorbents followed two fundamental steps. First, sorbents were selected which in theory have the capability to retain PGs from urine. Next, the different sorbents chosen were tested to evaluate their actual ability to selectively retain the PGs of interest (97).

The sorbents which were chosen for the study were determined by examining properties of the analyte (PGs) and the matrix (urine). First, the determination of the interactions which PGs could undergo was examined. Areas of carbon/hydrogen content with alkyl chains suggested that non-polar retention was probable. The presence of such polar groups

as hydroxyls (OH) and carbonyls (=O) indicated a potential for retention by polar interactions. Ionic interaction was indicated by the presence of the carboxylic acid moiety. However, this method was not evaluated for the analysis of PGs in urine due to the excessive quantities of compounds in urine which would undergo anionic and cationic interactions. Considering that PBA has been used for separation of the arachidonic acid metabolite, thromboxane B_2 (TXB_2), covalent interaction with some PGs appears possible due to the 1,3-diol present on the cyclopentane ring.

Next, the properties of the matrix and the potential interfering components which are contained in urine were considered. Urine is an aqueous media which contains many proteins, salts and solids. Components with polar and non-polar functionalities can be found throughout urine samples. This suggested that the interferences would undergo the same interactions with the sorbents as the PGs. Therefore, to determine which interactions would work most effectively, the sorbents required testing.

A sorbent testing scheme is shown in Figure 2-2 (97). First, each sorbent needed to be prepared. This was accomplished by washing the sorbent bed first and allowing the functional groups on the sorbent to interact with the solvent. The next step was to remove the wash solvent and create an environment that facilitated the analytes (PGs) retention. After this process, the testing procedure began and involved five steps.

Standards were prepared identical to a "real" sample and applied to the column (sorbent). The standards were then washed with the same solvent in which they were dissolved, and the eluent collected. The eluent was then checked for the presence of analyte, indicating sorbents which did not provide adequate retention of the analyte. Next, strong

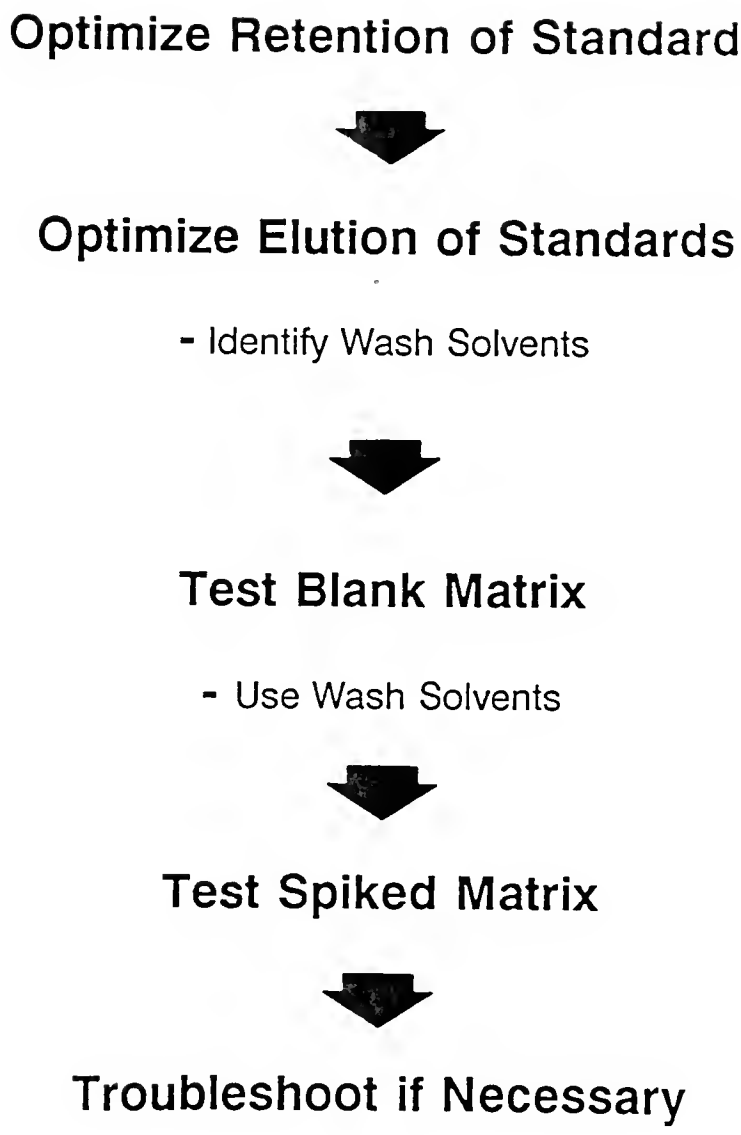


Figure 2-2: Sorbent testing scheme.

elution solvents were chosen of which small volumes can be utilized to completely elute the retained analyte. During this process, solvents which would not elute the analyte were identified for use as wash solvents. These were tested next with a blank matrix (urine) to determine the solvent(s) which produced the cleanest extract. Clearly, this was far more difficult to evaluate than the determination of analyte recovery. After developing a procedure which provided sufficient analyte retention and elution, as well as adequate clean-up of the matrix, the method was tested with a sample (urine) spiked with analyte. Recoveries found in this step were similar to those obtained with the standards. However, if problems had been encountered, either the sorbent, wash solvents or elution solvents may have been changed to provide for adequate retention and elution of the analyte in the matrix.

Antibody Affinity Extraction

Extraction methods for PGs based on liquid-liquid or solid-phase extraction are relatively nonselective and the final extracts are frequently unsuitable for direct analysis, even by highly specific GC/MS quantitation methods (7,101). The necessity of further purification of the extracts before chromatographic analysis makes the analytical procedures more complex, laborious and time-consuming to develop. This problem has been avoided in many cases by taking advantage of immunoadsorption techniques to simplify extraction and clean-up procedures for GC/MS analysis (18-21,102). Reports have shown that the selectivity of the immunoadsorption procedures may permit the direct analysis of extracts and eliminate the need for intermediate chromatographic clean-up.

Antibodies have been used for many years for the analysis of PGs by radio-immunoassay (RIA). Unfortunately, the presence of substances within the sample matrix which exhibit cross-reactivity with the polyclonal antibody can be considerable (39,103). For example, antibodies for 20 carbon PGs and their metabolites may also bind the corresponding dinor metabolites present in the matrix (19). Thus, HPLC is frequently employed as a separation technique prior to RIA to avoid cross-reactivity. Reports have shown that without separation of cross-reacting components by HPLC, PG levels have been found 20 times higher than the actual levels present (7,8). Immunoadsorption purification has been utilized as well prior to PG analysis by RIA (104,105). However, this method has the disadvantage of combining a purification procedure based on immunoaffinity with a measurement procedure based upon the same principle. The advantage of utilizing immunoadsorption for purification before GC/MS analysis is that the highly specific antibody will enhance the selectivity by providing discrimination which is unrelated and complements the characteristics of GC/MS. This results in an analysis method for PGs which has a higher degree of specificity than RIA.

These ideas have been incorporated in the sample preparation of PGs. The inherent selectivity of the antibody-antigen interactions has been exploited for PG analysis by Knapp and Vrbanac to obtain relatively pure sample extracts (20,78). The basic principle of antibody affinity extraction is displayed in Figure 2-3. In a typical affinity chromatographic separation, the antibody is coupled to a stationary phase (the most popular is agarose gel). The selectivity of affinity separations is based on the principle of "lock and key" binding which

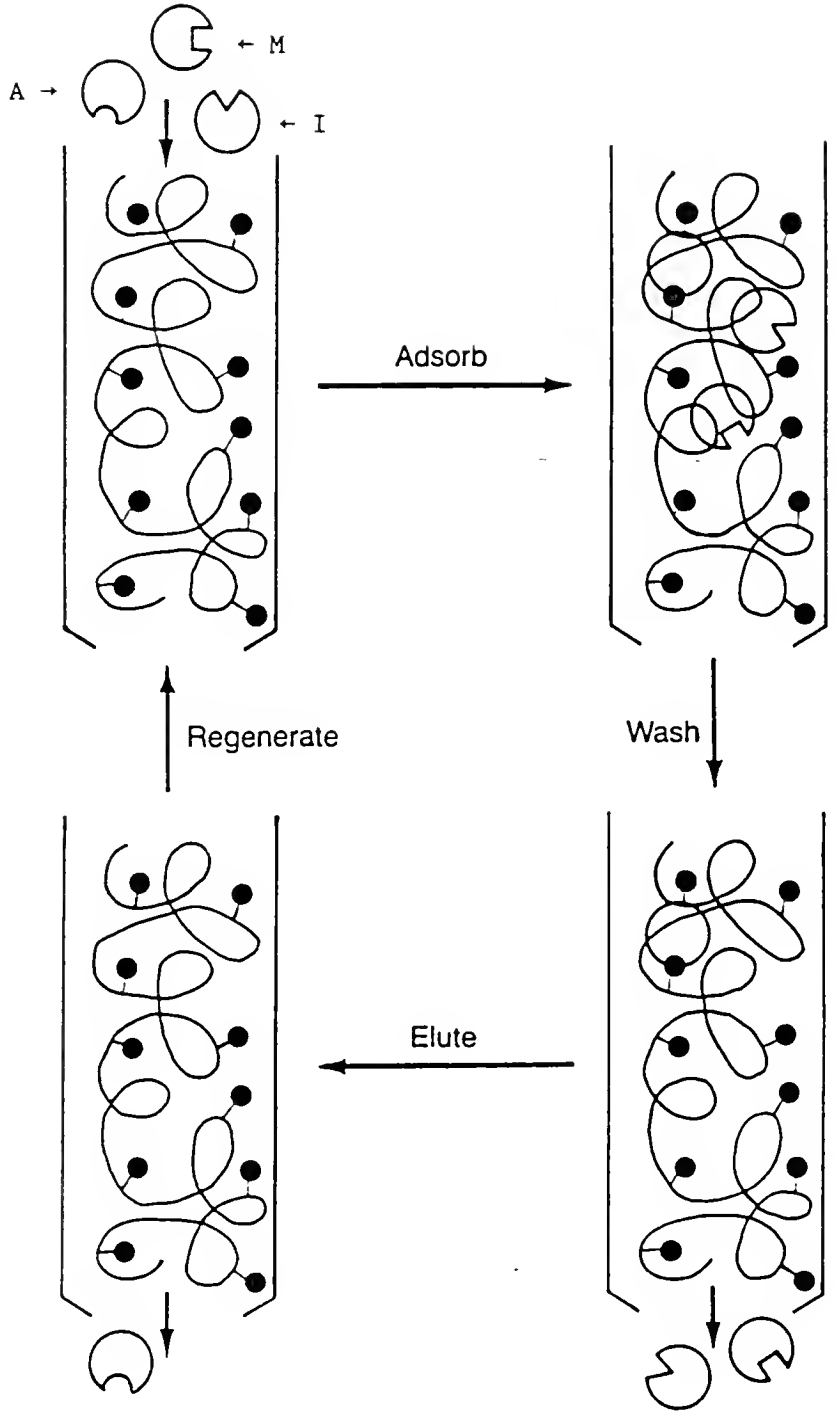


Figure 2-3: Basic principle of antibody affinity extraction: A = analyte; I & M = interferences.

occurs in biological systems. Extraction of the sample is accomplished by passing the solution (containing analytes and interferences) through the sorbent bed; the PGs which have affinity for the antibody are adsorbed while other components pass through unretained. The retained or adsorbed PGs are then eluted by changing the solvent.

Additional, secondary interactions are possible with immunoaffinity chromatography. One important interaction discussed earlier is due to the cross-reactivity of the polyclonal antibodies. Furthermore, non-specific binding of interfering components may occur during the immunoadsorption procedure. The bulk protein carrying the antibody has the potential for interaction of components in the sample matrix. In addition, polar interactions are possible between the silica stationary phase and any polar functionalities found in the sample matrix.

In general, antibody affinity purification can decrease significant loss of sample which can occur in TLC and HPLC. This method of sample preparation is relatively rapid and requires no additional purification of biological samples to obtain an adequate interference free GC trace. The greatest advantage is the significant selectivity of the separation process for antibody affinity purification compared to other conventional chromatographic methods.

Concepts for Derivatization

Derivatization of PGs has been important in their analysis by GC/MS, both to increase their volatility for GC separation and to provide for efficient EC-NCI to increase sensitivity of the GC/MS method. Many different derivatives have been used in the analysis for PGs (10,11). As reported earlier in Chapter 1, the most commonly used derivative for

quantitative analysis by GC/MS is the methoxime/pentafluorobenzyl/trimethylsilyl (MO/PFB/TMS) derivative.

The keto group on PGE_2 is converted to the methoxime derivative to prevent silylation which can interfere with quantitation by producing additional derivatives. Pentafluorobenzyl (PFB) esters are created to enhance the efficiency of ionization by EC-NCI in order to achieve low level determinations of PGs. These PFB esters have been found to give about twice the sensitivity of the methyl ester derivative (106). Reaction times are fast (~20 min) and quantitative (~100%) for this derivatization procedure. The hydroxyl groups are converted to trimethylsilyl (TMS) ethers using O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). This TMS donor has the additional advantage of creating extremely volatile reaction by-products which usually elute with the solvent front in the GC trace.

Even though the derivatization for quantitative analysis of PGs by GC/MS has been thoroughly documented, there are many variations in the literature. It has been reported that by performing the methoximation before the esterfication a fivefold increase in the derivative yield can be obtained (47). However, many researchers still perform the esterfication step first in the derivatization procedure (10,11,74). Reaction times for the methoximation step vary in the literature ranging from one hour at 60° to 24 hours at room temperature. These differences, in addition to the comparison of techniques for the removal of excess derivative reagents by liquid-liquid extraction and nitrogen evaporation were explored in this study.

Experimental

Prostaglandins and Reagents

All solvents were reagent or HPLC grade. Prostaglandin E_2 (PGE_2) was purchased from Sigma Chemical Co. (St. Louis, MO). [5,6,8,11,12,14,15 - $^3H_2$]-PGE_2 and Riafluor liquid scintillator were from New England Nuclear (Boston, MA) and were a gift from Dr. J. Neu of the Department of Pediatrics, University of Florida (Gainesville, FL). The solid-phase extraction columns were purchased from Analytichem International, Inc. (Harbor City, CA) and Waters Assoc. (Sep-Pak columns; Milford, MA). 3,3',4,4'-($^2H_4$) PGE_2 and the antibody affinity sorbent were gifts from Drs. J.J. Vrbanac and D.R. Knapp of the Department of Pharmacology, Medical University of South Carolina (Charleston, SC). The derivatization reagents and solvents pyridine, O-methylhydroxylamine hydrochloride, acetonitrile, and N,N-diisopropylethyl amine for GC/MS percent recovery studies were all purchased from Sigma Chemical Co.. Pentafluorobenzyl-bromide (PFBBr) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Pierce Chemical Co. (Rockford, IL). Urine was obtained from the author. All glassware was silanized with a solution of 5% dimethyldichlorosilane in toluene. These two chemicals were both purchased from Sigma Chemical Co.. Helium used as GC carrier gas and methane (>99%) used as the chemical ionization reagent gas were from Matheson Gas Products, Inc. (Orlando, FL).

Sample Preparation

The sorbents for the percent recovery studies were chosen and tested according to the procedures discussed earlier in this chapter. Extraction

procedures were determined for the non-polar, polar and phenyl boronic acid columns by detection of the tritium-labeled PGE_2 by scintillation counting. The sorbents chosen are listed with the final extraction procedure used for the percent recovery studies for both scintillation counting and GC/MS.

Non-polar columns: octyl (C8), octadecyl (C18) and phenyl (PH)

(1) Conditioned the column with 10 mL of HPLC water and 10 mL of methanol.

(2) Passed solution of PGE_2 (acidified to pH 3.5 with formic acid) through the column.

(3) Washed the column with 10 mL of HPLC water and 10 mL petroleum ether.

(4) Eluted PGE_2 with 10 mL of ethyl acetate.

Polar columns: silica (SI), cyanopropyl (CN), aminopropyl (NH_2) and diol (2OH)

(1) Conditioned the column with 10 mL of benzene:ethyl acetate (80:20 volume:volume).

(2) Passed solution of PGE_2 (acidified to pH 3.5 with formic acid) through the column.

(3) Washed the column with 10 mL benzene:ethyl acetate (60:40 v:v).

(4) Eluted PGE_2 with 10 mL benzene:ethyl acetate:methanol (60:40:30 v:v:v).

Phenyl boronic acid column (PBA)

(1) Conditioned the column with 5 mL of 0.1 M hydrochloric acid and 5 mL of 0.1 M sodium hydroxide.

(2) Passed sample of PGE_2 (adjusted to pH 8.5 with 0.1 M phosphate buffer (PBS)) through the column.

(3) Washed the column with 5 mL of methanol and 5 mL of HPLC water.

(4) Eluted PGE_2 with 5 mL of 0.1 M PBS (pH 6.5).

The antibody affinity columns were tested and percent recovery data calculated only with GC/MS.

Antibody affinity column [Immunoaffinity (IA)]

(1) Conditioned the column with 20 mL of PBS (pH 7.4).

(2) Passed solution of PGE_2 (acidified to pH 3.5 with formic acid) through the column.

(3) Allowed the sample to settle into the sorbent bed for 15 min at room temperature.

(4) Washed the column with 25 mL of PBS (pH 7.4) and 10 mL HPLC water. Removed all remaining water in the column.

(5) Eluted PGE_2 with 15 mL of 95% acetonitrile solution (v:v).

(6) Washed column with an additional 10 mL of 95% acetonitrile to assure removal of all the PGE_2.

(7) Immediately rinsed the column with 10 mL of HPLC water and 15 mL of PBS (pH 7.4).

Scintillation Counting

A stock solution of ^3H-PGE_2 was used for the percent recovery studies. This solution was 0.09375 microcuries (μCi)/microliter (μL) and had a specific activity of 169.5 μCi/millimole. Six microliters of this original solution was diluted with 100 μL of absolute ethyl alcohol creating a solution of 5.625×10^{-3} μCi/μL. Ten microliters of this

standard solution, corresponding to 3.319×10^{-4} mmoles or 0.1218 mg was passed through each column tested. Following the extraction procedures the eluent was collected and the solvent evaporated with nitrogen. The ^{3}H-PGE_2 was then diluted with 100 μL of PBS (pH 7.4). Additionally, 3.5 mL of Riafluor liquid scintillator were added to the 9.375×10^{-3} μCi solution of ^{3}H-PGE_2 before the counting process. Each extraction was performed three times with three individual columns.

A calibration curve was prepared in the same manner with the exception of the actual extraction step (Figure 2-4a). Aliquots of 4, 5.5, 8, 10.5, 12 and 13.5 microliters of the 5.625×10^{-3} μCi/μL solution were added to separate vials and each diluted with 100 μL of the PBS (pH 7.4). In addition, a blank containing only 100 μL of the PBS (pH 7.4) was prepared. The Riafluor liquid scintillator was added and the standards counted and used to calculate the percent recovery values for the different columns tested.

Gas Chromatography/Mass Spectrometry (GC/MS)

Ten nanograms (ng) of PGE_2 were passed through each column for the percent recovery studies of standards. Following the extraction procedure for the columns tested, 10 ng of $^{2}H_4$-PGE_2 were added and the solutions were evaporated to dryness with nitrogen. The same procedure was followed for extraction of PGE_2 in urine for percent recovery studies except that the 10 ng of PGE_2 added to the urine is in addition to the endogenous levels present.

Calibration curves for the GC/MS analysis were prepared by adding a constant amount of $^{2}H_4$-PGE_2 (25 ng) and increasing amounts of PGE_2 in the

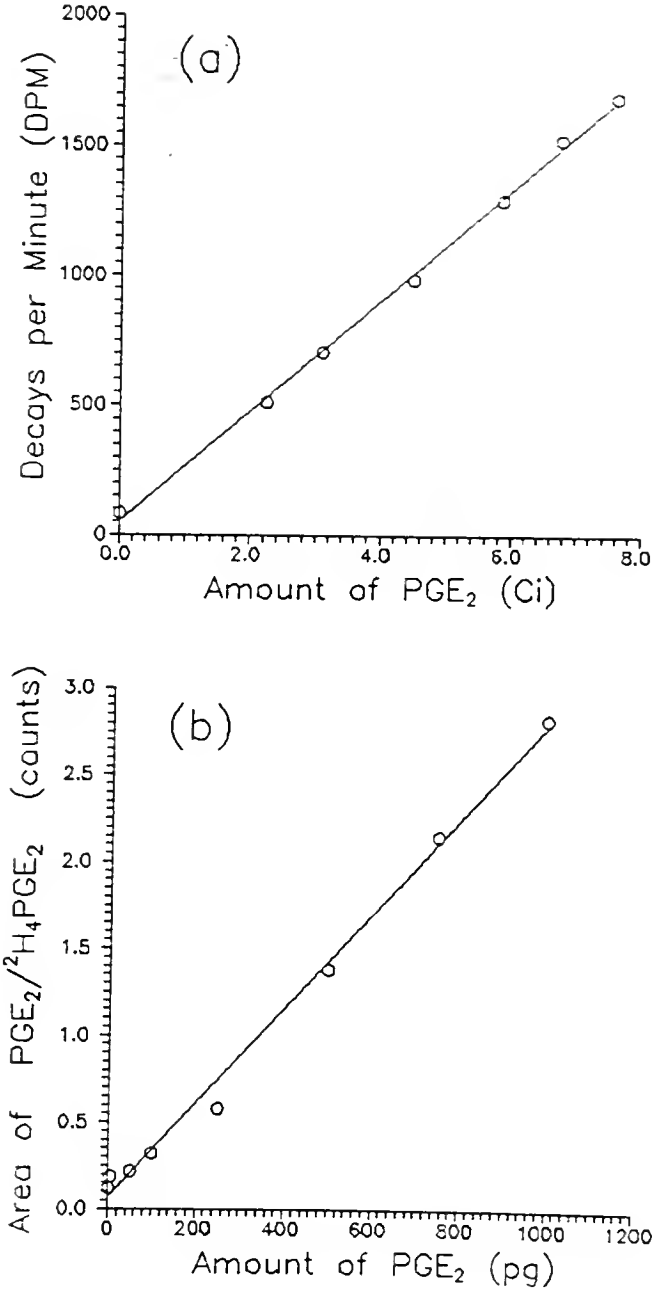


Figure 2-4: Calibration curves: (a) Scintillation counting (b) Gas chromatography/mass spectrometry (GC/MS) with selected-ion monitoring (SIM).

Table 2-1: Calibration Curve Dilutions for GC/MS

$^2H_4$-PGE_2 Added (ng)	PGE_2 Added (ng)	Volume of Dilution (μL)	Concentration of $^2H_4$-PGE_2 (pg/μL)	Concentration of PGE_2 (pg/μL)
25.0	0.0	50.0	500.0	0.0
25.0	0.25	50.0	500.0	5.0
25.0	2.5	50.0	500.0	50.0
25.0	5.0	50.0	500.0	100.0
25.0	12.5	50.0	500.0	250.0
25.0	25.0	50.0	500.0	500.0
25.0	37.5	50.0	500.0	750.0
25.0	50.0	50.0	500.0	1000.0

solution (Figure 2-4b). Table 2-1 lists the amounts of $^2H_4$-PGE_2 and standard PGE_2 added to each vial and the final concentrations after dilution with 50 μL of silanizing reagent.

Derivatization for GC/MS

The methoxime/pentafluorobenzyl ester/trimethylsilyl (MO/PFB/TMS) derivatives were formed for the GC/MS percent recovery and derivatization studies. The method used was similar to the derivatization of H. L. Hubbard et al. (19). The standards and samples of $^2H_4$-PGE_2 and PGE_2 after evaporation were treated with 100-200 μL of methylhydroxylamine HCl in dry pyridine (4 mg/mL), allowed to stand overnight at room temperature, then evaporated under nitrogen until dry. Each sample was acidified by adding 200 μL of 1N formic acid, extracted with two 1 mL aliquots of ethyl acetate, and the extract dried under nitrogen. Then 50 μL of acetonitrile, 30 μL of 30% PFBBr in acetonitrile, and 15 μL of 10% N,N-diisopropylethylamine in acetonitrile were added to the dried methoxime derivative. Each solution was allowed to stand for 30 minutes at room temperature before the reagents were evaporated with nitrogen. Excess derivatizing reagent was removed by dissolving the sample in 200 μL of distilled water and extracting with two 1 mL aliquots of a methylene chloride:hexane (50:50 v:v) solution; the extract was then dried under nitrogen. The trimethylsilyl derivative then was formed by adding 50 μL of BSTFA to the standards for the calibration curve and 20 μL to the extraction samples and allowing the solutions to stand overnight at room temperature. One-microliter injections containing 500 pg of $^2H_4$-PGE_2 were made of each standard and sample.

Instrumentation

A Beckman LS 3800 liquid scintillation counter and a Finnigan MAT triple stage quadrupole (TSQ45) gas chromatograph/mass spectrometer were used in these studies. Gas chromatography was carried out on a conventional J&W Scientific (Folsom, CA) DB-1 (30 m long, 0.25 mm i.d., 0.25 μm film thickness) capillary column in the splitless mode with helium carrier gas at a flow rate of 41 cm/s (inlet pressure 18-20 psi). The initial temperature of 250°C was held for 30 s, then increased at 20°C/min to 310°C for the calibration curve and percent recovery studies of standards. Urine percent recovery data were obtained with an initial temperature of 100°C held for 30 s, increased at 25°C/min to 250°C, then increased again at 5°C/min to 310°C.

Mass spectrometry conditions were: interface and transfer line temperature 300°C, ionizer temperature 190°C, electron energy 100 eV and emission current 0.3 mA. Electron-capture negative chemical ionization (EC-NCI) was carried out with methane at an ionizer pressure of 0.45 torr.

In the GC/MS percent recovery and derivatization studies, a specific ion for PGE_2 (524^-; $[MO/TMS\text{-}PFB]^-$) and for $^2H_4\text{-}PGE_2$ (528; $[MO/TMS\text{-}PFB]^-$) were selected and monitored throughout these studies. The selected ion monitoring mode (SIM) with quadrupole one was used on the mass spectrometer. A baseline was chosen visually on the GC trace and the areas for PGE_2 and $^2H_4\text{-}PGE_2$ calculated by the INCOS computer system for the calibration curve and percent recovery samples. The area of PGE_2 divided by the area of $^2H_4\text{-}PGE_2$ in the standards gives a ratio which is used in the calibration curve. The amount of PGE_2 recovered through each column was

calculated by comparing the ratio of these ions after the extraction procedure to that of the calibration curve.

Results and Discussion

Percent Recovery Studies (Scintillation Counting)

Table 2-2 shows the percent recovery results for the different sorbents tested. These recoveries were determined by scintillation counting of ^{3}H-PGE_2 standards. Three samples were extracted for each sorbent and the average and percent relative standard deviation (%RSD) calculated. Examination of Table 2-2 indicates that a wide range of recoveries was found for the different sorbents tested.

The non-polar sorbents investigated were octyl (C8), octadecyl (C18) and phenyl (PH). The C18 columns had the highest percent recoveries (97.2%) with the C8 (81.8%) and the PH (8.8%) columns having lower recoveries. These low recovery values for the C8 and PH columns signify either: (1) PGE_2 was unretained as the initial standard solution was passed through the column; (2) PGE_2 was eluted during the wash procedure; or (3) PGE_2 was irreversibly bound to the column or not effectively eluted. The reasons for poor recovery were investigated by examining all the eluents which were passed through the respective columns to determine the presence of PGE_2. It was discovered that the C8 and PH columns did not initially retain PGE_2. Octadecyl columns exhibited the smallest variation from column to column with a %RSD of 0.6.

Polar sorbents tested were silica (SI), cyanopropyl (CN) aminopropyl (NH_2) and diol (2OH). As with the non-polar columns, a wide range of recoveries were discovered for the different polar interactions tested.

Table 2-2: % Recovery of Standard PGE_2 by Scintillation Counting

Column	% Recovery Data			Average % Recovery	%RSD[a]
	Column #				
	1	2	3		
C8 (Octyl)[b]	80.11	82.17	83.08	81.8	1.9
C18 (Octadecyl)	97.91	96.99	96.84	97.2	0.6
PH (Phenyl)	8.35	9.20	8.78	8.8	4.8
SI (Silica)	97.89	100.03	103.01	100.4	2.5
CN (Cyanopropyl)	24.02	27.92	22.82	24.9	10.7
NH_2 (Aminopropyl)	12.75	13.96	11.12	12.6	11.3
2OH (Diol)	89.19	93.44	98.55	93.7	5.0
PBA (Phenyl Boronic Acid)	40.72	38.23	41.08	40.0	3.9

[a] % Relative Standard Deviation
[b] All columns were purchased from Analytichem.

The two sorbents that exhibited greater than 90% recovery were the SI (100.4%) and 2OH (93.7%). The recoveries for these columns were found to be much higher than those found for CN (24.9%) and NH_2 (12.6%) columns. This difference in recoveries may be attributed to the interaction of the polar groups on PGE_2 (particularly the hydroxyls) with the hydroxyl groups on the SI and 2OH, rather than with the carbon/nitrogen interaction with CN or the amine group with the NH_2. Investigation of the eluents showed that PGE_2 was not effectively retained initially for the CN and NH_2 columns. The variation from column to column (%RSD) for the SI (2.5%) and 2OH (5.0%) were less than that found for CN (10.7%) and NH_2 (11.3%).

In addition, phenyl boronic acid columns were tested. The percent recovery found using this type of interaction was 40.0%, well below the recovery values found for C18, SI and 2OH columns. This extraction technique is based on the premise that the tetrahedral anionic form of boronates condense with 1,2- or 1,3-diols to form five- or six- membered covalent complexes (107). The low recovery of PGE_2 observed for the PBA column can possibly be explained by the inability of the boronate to condense with the diols on the cyclopentane ring of PGE_2 to form a stable complex. An explanation for the inability of PGs to condense with the PBA column was reported by Lawson et al. (17). They believe that the tendency of the planar phenyl groups to orient so that their pi (π) orbitals align or are stacked, thereby forcing the boronic acid groups to be too close together, not allowing the sterically fixed cyclic 1,3-diols on PGs free access.

Data obtained from testing these sorbents suggests that PGE_2 has preference for retention on specific non-polar (C18) and polar (SI and

20H) sorbents. Even sorbents with the same type of interactions demonstrate varied retention for PGE_2. Recoveries for the C18, SI and 20H columns are similar and demonstrate adequate retention (>90%) of PGE_2 to justify further investigation.

Percent Recovery Studies (GC/MS)

Percent recovery data for standard PGE_2 by GC/MS is listed in Table 2-3. The columns tested in this study were those that had been found to provide adequate retention (>90%) for PGE_2 in the previous scintillation counting experiments. In addition, this study shows recovery data for another brand of octadecyl sorbent (Sep-Pak) and a very selective sorbent using antibody-antigen interaction (immunoaffinity). As in the initial recovery study (Table 2-2), three individual columns were each used to extract three samples of standard PGE_2. Three injections of each sample were made into the GC/MS. The average of the three injections and the three samples, in addition to the %RSD is listed in Table 2-3.

Comparing the recovery data in Table 2-2 to the data in Table 2-3, the average recovery values for the C18, SI and 20H are similar. The values determined by GC/MS are consistently 3-10% lower than those determined by scintillation counting. However, this slight difference could be attributed to the basic difference in calculating counting data and areas of GC/MS. The variation between columns is again small (<5.0%) for all sorbents tested. The immunoaffinity column demonstrated a percent recovery (93.1%) quite adequate for retention of PGE_2. Octadecyl columns from two different suppliers were compared to examine differences in retention and selectivity. The recovery data for the Sep-Pak (Waters)

Table 2-3: % Recovery of Standard PGE_2 by Gas Chromatography/Mass spectrometry

Column	Column #	% Recovery Data GC Injection # 1	2	3	Average % Recovery	%RSD[a]	Average % Recovery of 3 Columns	%RSD[a] of 3 Columns
	1	91.85	91.72	91.15	91.6	0.4		
Sep-Pak	2	86.30	84.60	84.12	85.0	1.4	89.1	4.0
(Octadecyl)	3	89.01	92.63	90.09	90.6	2.1		
	1	89.43	89.59	92.67	90.6	2.0		
C18	2	95.63	94.98	95.06	95.2	0.4	92.8	2.5
(Octadecyl)	3	92.51	91.82	93.17	92.5	0.7		
	1	96.52	93.02	96.09	95.2	1.9		
SI	2	82.04	94.11	92.21	89.5	7.3	90.8	4.3
(Silica)	3	86.29	86.79	90.49	87.9	2.6		
	1	92.35	91.76	92.07	92.1	0.3		
2OH	2	90.44	88.69	91.13	90.1	1.4	90.0	2.4
(Diol)	3	88.58	86.90	87.88	87.8	1.0		
	1	88.50	98.18	102.02	96.2	7.2		
IA	2	91.11	87.88	88.34	89.1	2.0	93.1	3.9
(Immununoaffinity)	3	90.67	89.55	101.60	93.9	7.1		

[a] % Relative Standard Deviation

columns were slightly lower than that found for the C18 (Analytichem) columns. This may be attributed to experimental error in the extraction procedure; however, reports suggest that Sep-Paks have considerable faults compared to octadecyl sorbents from other manufacturers (108).

Table 2-4 contains the percent recovery results of standard PGE_2 spiked into urine by GC/MS analysis. The columns tested in this study were C18, SI, 2OH and a combination of C18 plus immunoaffinity (IA). Biological samples can be directly applied to the IA column; however, for PG analysis Knapp and Vrbanac (78) have found an advantage in preceeding the IA purification procedure with a C18 column extraction. The advantage is that employing the C18 extraction first removes large concentrations of extremely polar impurities found in urine which can non-specifically bind to the IA column. Averages and %RSD are listed in the table for three injections of each sample and the three extractions which were performed on individual columns. The data indicate that the columns (SI and 2OH) which utilize polar interactions are not effective for retention of PGE_2 in urine, even though they were successful for PGE_2 standards. This is presumably due to competition for binding sites on the sorbent between matrix components in the urine and PGE_2. The non-polar C18 column and the C18 column coupled with the IA column provided similar recoveries for PGE_2 in urine as in standards. The variation from column to column is low for all cases (<7%) including the C18 and IA samples. In the case of the IA column data, the same sorbent bed (or column) was used for all three samples. The %RSD for the three samples, in addition to the three average recovery values, demonstrate the reusability of the IA sorbent. The slight decrease in the recovery values between samples 1-3 indicates

Table 2-4: % Recovery of Standard PGE_2 in Urine by Gas Chromatography/Mass Spectrometry

Column(s)	Column #	% Recovery Data GC Injection # 1	2	3	Average % Recovery	%RSD[a]	% Recovery of 3 Columns	%RSD[a] of 3 Columns
	1	89.95	84.13	88.66	87.9	3.5		
C18	2	96.88	99.66	99.16	98.6	1.5	92.1	6.3
(Octadecyl)	3	90.74	90.33	89.20	90.1	0.9		
	1	9.58	10.22	10.88	10.2	6.4		
SI	2	10.35	10.81	10.51	10.6	2.2	10.6	4.0
(Silica)	3	10.67	10.91	11.62	11.1	4.5		
	1	23.27	18.61	26.82	22.9	18.0		
2OH	2	21.29	24.48	23.82	23.2	7.3	22.2	6.8
(Diol)	3	21.09	20.46	19.81	20.5	3.1		
	1	81.25	93.65	102.7	92.5	11.6		
C18 + IA[b]	2	87.15	92.08	87.46	88.9	3.1	89.7	2.8
	3	87.50	88.65	86.97	87.7	1.0		

[a] % Relative Standard Deviation
[b] Three different C18 columns, but the only one IA column.

that no carry-over of PGE_2 occurred from sample to sample. Thus, the IA sorbent can be reused for many urine samples in combination with a C18 column without loss of affinity for PGE_2. The ability of IA to effectively separate urine matrix components from PGE_2 will be discussed in a later chapter.

Derivatization Studies

Table 2-5 lists the results of the study of different derivatization procedures. The GC/MS peak areas for three samples are listed along with their average and %RSD. Each sample injected onto the GC column contained 500 pg of PGE_2. Comparing the different results, the most effective method of derivatization can be determined.

The first method listed followed the derivatization procedure discussed in the experimental section. Comparing that method to a second method, in which a more rapid methoximation at an elevated temperature (1 hr at 60°C) was used, the peak area of method one was 1.4 times greater when the 24 hour methoximation was employed. This suggests that at longer reaction times more complete methoximation occurs. Recently, a study of the methoximation of various PGs was reported in the literature (109). These results showed that efficient methoximation of PGE_2 by a procedure similar to method one was 1.1 times greater than method two with a %RSD of 11.8%. This corresponds to the values which were obtained in this study. Another question addressed by this study is whether to perform the methoximation step before or after the PFB esterification. Examining the peak areas obtained for method three and comparing them to method one, similar areas were calculated for 500 pg. The data suggest that either

Table 2-5: Study of Different Derivatization Procedures for PGE2 by GC/MS with SIM for the $[M\text{-}PFB]^-$ ion

	Peak Area of PGE_2 (counts)				
Method	Sample 1	Sample 2	Sample 3	Average	%RSD[a]
1[b]	1.64×10^6	2.21×10^6	2.01×10^6	1.95×10^6	14.8
2[c]	1.29×10^6	1.58×10^6	1.44×10^6	1.44×10^6	10.1
3[d]	2.16×10^6	1.74×10^6	2.00×10^6	1.97×10^6	10.8
4[e]	1.14×10^6	1.14×10^6	1.16×10^6	1.15×10^6	1.0
5[f]	1.21×10^6	1.14×10^6	1.12×10^6	1.15×10^6	4.1

[a] % Relative Standard Deviation

[b] Procedure described on page 41

[c] Methoximation for 1 hr at 60°C

[d] Esterification performed before methoximation

[e] Nitrogen evaporation only after the methoximation step

[f] Nitrogen evaporation performed after all steps (no liquid-liquid extraction was performed)

step (methoximation or the PFB esterification) can be performed first. The other question proposed in this study was the use of liquid-liquid extraction to remove excess derivatizing reagents or the simpler, more rapid method of only nitrogen evaporation. Two methods were studied, one in which only nitrogen evaporation was performed after the methoximation step, then liquid-liquid extraction after the esterification (method 4) and the other in which only nitrogen evaporation was performed after all derivatization steps (method 5). Comparing these two methods with method one, the areas for 500 pg for both method four and five were approximately 40% less than method one. This suggests that removal of the excess derivatizing reagents by liquid-liquid extraction is essential prior to GC/MS, despite the increase in sample preparation time. The concentration of PGE_2 in urine is 100 to 400 pg/mL, thus having an effective derivatization procedure which enhances the sensitivity of PGE_2 is vital, even at the expense of additional analysis time.

Conclusions

The optimum sample preparation for PGE_2 in urine has been determined in this chapter. Extraction and purification procedures as well as derivatization steps have been investigated. The results from recovery studies show that the use of either a C18 column or a combination of C18 and IA columns achieve adequate quantitative recoveries for PGE_2 in urine. Derivatization study results indicate that the time and temperature of the methoximation reaction are important and appears to be optimum at longer reaction times with lower temperatures. Performing either methoximation or PFB esterification first in the derivatization procedure has little

effect on the area calculated for PGE_2. Results from this study demonstrate the advantage of using liquid-liquid extraction methods to remove the excess derivatization reagents after each step in the derivatization procedure.

CHAPTER 3

OPTIMIZATION OF GC/MS AND GC/MS/MS CONDITIONS FOR TRACE DETERMINATION OF PROSTAGLANDINS

Introduction

Many reports of GC/MS analysis of prostaglandins (PGs) can be found in the literature (2,17,48,62,101). The conditions employed in each case vary depending on the type of analysis (qualitative or quantitative), sample matrix (urine, serum, etc.), and targeted concentration. In the trace determination of PGs, optimization of conditions is critical. Detection of low levels of PGs (100-400 pg/mL) in urine requires a technique that is both sensitive and selective. The many parameters which exist in GC/MS and GC/MS/MS can be varied according to the analysis to enhance either sensitivity or selectivity. Thus, to achieve the proper conditions for trace determination of PGs the various parameters must be characterized and optimized.

Experimental

Prostaglandins and Reagents

All solvents were reagent or HPLC grade. Prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was purchased from Sigma Chemical Co. (St. Louis, MO). The derivatization reagents pyridine, O-methylhydroxylamine hydrochloride, acetonitrile and N,N-diisopropylethyl amine were all

purchased from Sigma Chemical Co. Pentafluorobenzylbromide (PFBBr) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Pierce Chemical Co. (Rockford, IL). All glassware was silanized with a solution of 5% dimethyldichlorosilane in toluene. These two chemicals were both purchased from Sigma Chemical Co. Helium used as GC carrier gas, methane (>99%) as chemical ionization reagent gas and nitrogen, argon and xenon used as collision gases were from Matheson Gas Products, Inc. (Orlando, FL).

Derivatization

The MO/PFB/TMS derivative of PGE_2 and the PFB/TMS derivative of $PGF_{2\alpha}$ were prepared by the same procedure as in Chapter 2.

Instrumental Conditions

A Finnigan MAT triple stage quadrupole (TSQ45) gas chromatograph/mass spectrometer was employed. Mass spectrometry conditions were: interface and transfer line temperature 300°C, ionizer temperature 190°C, electron energy 100 eV and emission current 0.3 mA. GC was carried out on a short J&W Scientific (Folsom, CA) DB-1 (3 m long, 0.25 mm i.d., 0.25 μm film thickness) capillary column in the splitless mode with helium carrier gas at an inlet pressure of 4-6 psi. The initial temperature of 200°C was held for 30 s, then increased at 20°C/min to 260°C. The injector temperature was 300°C.

Both full scan mass spectra and selected-ion monitoring (SIM) were used in the GC/MS studies. An electron multiplier (EM) setting of 900 V was used for the full scan spectra and a preamp gain of 10^{-8} A/V. A baseline was chosen visually on the GC trace and the area for PGE_2 (524^-)

and $PGF_{2\alpha}$ (569^-) calculated by the INCOS computer system in the SIM mode of operation. In GC/MS/MS optimization studies full daughter spectra and selected-reaction monitoring (SRM) were employed and areas calculated by the same method as described for GC/MS. The EM was set at 1500 V for the full daughter spectra obtained and a preamp gain of 10^8 V/A. In the GC/MS/MS optimization the $[M\text{-}PFB]^-$ carboxylate anions of PGE_2 and $PGF_{2\alpha}$ were selected in the first quadrupole (Q1) region and passed into the collision cell (Q2). In this region these ions underwent CAD to form characteristic fragments which were then mass analyzed in the third quadrupole (Q3). A full daughter spectrum was acquired over the mass range of 100-600 amu.

Calibration curves were prepared for both GC/MS and GC/MS/MS after optimization of the various parameters. Selected-ion monitoring and selected-reaction monitoring were used to determine linearity, precision and limits of detection for standard PGE_2 utilizing GC/MS and GC/MS/MS. The EM was set at 1700 V for both the SIM and SRM calibration curve data with a preamp gain of 10^8 V/A.

Mass Spectrometry (GC/MS)

Choice of the appropriate ionization method is essential for trace determination of PGs by GC/MS. Electron ionization (EI) has been reported in the determination and identification of various PGs (42,43,57). Structural information is obtained by this technique due to the abundance of fragment ions which are produced. However, in trace analysis of PGs the creation of a single ion with a maximized intensity is preferred.

Chemical ionization (CI) has the advantage of usually producing few fragment ions and a very intense molecular ion. Many reports of chemical ionization GC/MS for PG analysis appear in the literature (65-72). Both positive and negative CI have been incorporated for PG analysis. The literature reports that appropriate derivatization of PGs coupled with electron-capture negative chemical ionization (EC-NCI) results in the detection of low levels of PGs (69-72). These reports generally employ methane (CH_4) as a reagent gas for its ability to thermalize electrons. Thus, both the GC/MS and GC/MS/MS optimization studies have utilized EC-NCI with methane as the reagent gas.

Ionizer Pressure Study

Although the literature includes numerous examples of methane as a reagent gas for EC-NCI, many dramatically different ion source pressures have been employed. This study was performed to determine the optimum ionizer pressure at which the $[M\text{-}PFB]^-$ ions of PGE_2 and $PGF_{2\alpha}$ are produced in the ion source. Figure 3-1 shows the average areas determined at different ionizer pressures of methane for the 524^- ion (PGE_2) and 569^- ion ($PGF_{2\alpha}$). The average of three one-microliter injections of a 100 pg/μL solution of both PGE_2 and $PGF_{2\alpha}$ have been plotted on the graph. The optimum ion source pressure for PGE_2 and $PGF_{2\alpha}$ occurs at 0.50 Torr of methane.

At ionizer pressures lower than 0.50 Torr the $[M\text{-}PFB]^-$ ion has a lower percent relative intensity compared to the reconstructed ion current (RIC) for both PGE_2 and $PGF_{2\alpha}$. As the ion source pressure is gradually increased above 0.50 Torr, fragment ions begin to increase in relative

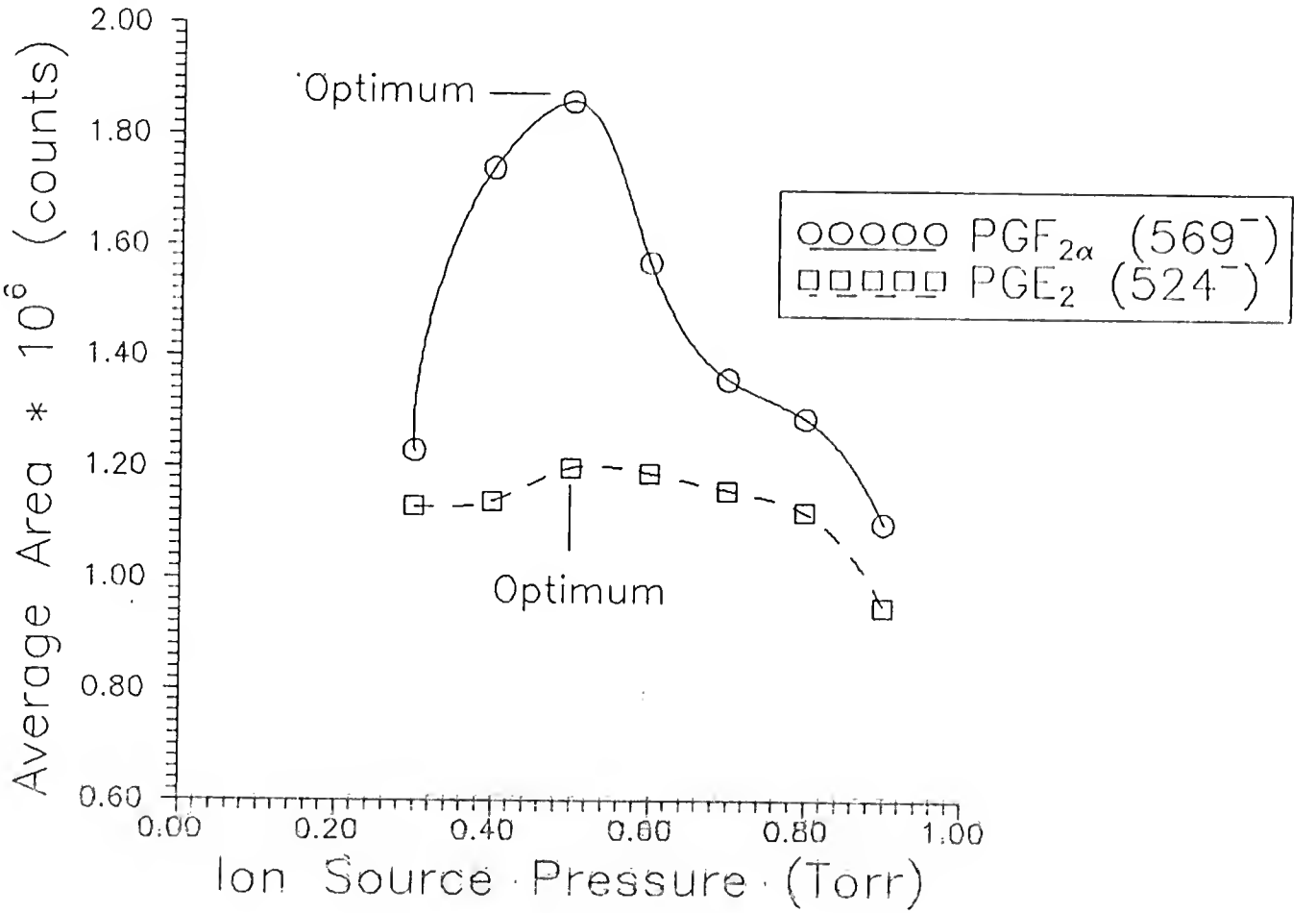


Figure 3-1: Ion source pressure study of the [M-PFB]⁻ ion of PGE_2 and $PGF_{2\alpha}$.

intensity and contribute more to the RIC, thus decreasing the relative intensity of the $[M-PFB]^-$ ion compared to the RIC.

Ionizer Temperature Study

Reports in the literature have cited ion source temperatures for EC-NCI/GC/MS and EC-NCI/GC/MS/MS in the range of 110°C to 200°C (69-78). Figure 3-2 indicates the optimum ion source temperature observed in this study for the analysis of PGE_2 and $PGF_{2\alpha}$. Selected-ion monitoring (SIM) of the 524^- (PGE_2) and 569^- ion ($PGF_{2\alpha}$) was used over a range of ion source temperatures from 100°C to 190°C. Three one-microliter injections of a 500 pg/μL solution of PGE_2 and $PGF_{2\alpha}$ were performed at ten different ion source temperatures. The average of the three injections is plotted on the graph. Both PGE_2 and $PGF_{2\alpha}$ have an optimum ion source temperature at 190°C. Thus, the $[M-PFB]^-$carboxylate anion of PGE_2 and $PGF_{2\alpha}$ optimize at the maximum ion source temperature of the instrument.

The percent relative intensity of the $[M-PFB]^-$ ion compared to the RIC increases with an increase in the ion source temperature and reaches a maximum at 190°C. In addition fragment ions increase, as the temperature is elevated to about 140°C to 150°C, then these fragments gradually decrease as the ion source temperature is raised above 150°C. These two observations lead to the optimum ion source temperature of 190°C for the $[M-PFB]^-$ ion.

Electron-Capture Negative Chemical Ionization Mass Spectra

The mass spectra of standard PGE_2 and $PGF_{2\alpha}$, obtained at the optimum ion source pressure and temperature, are shown in Figure 3-3a and Figure 3-3b. Both spectra demonstrate the advantage of employing EC-NCI for

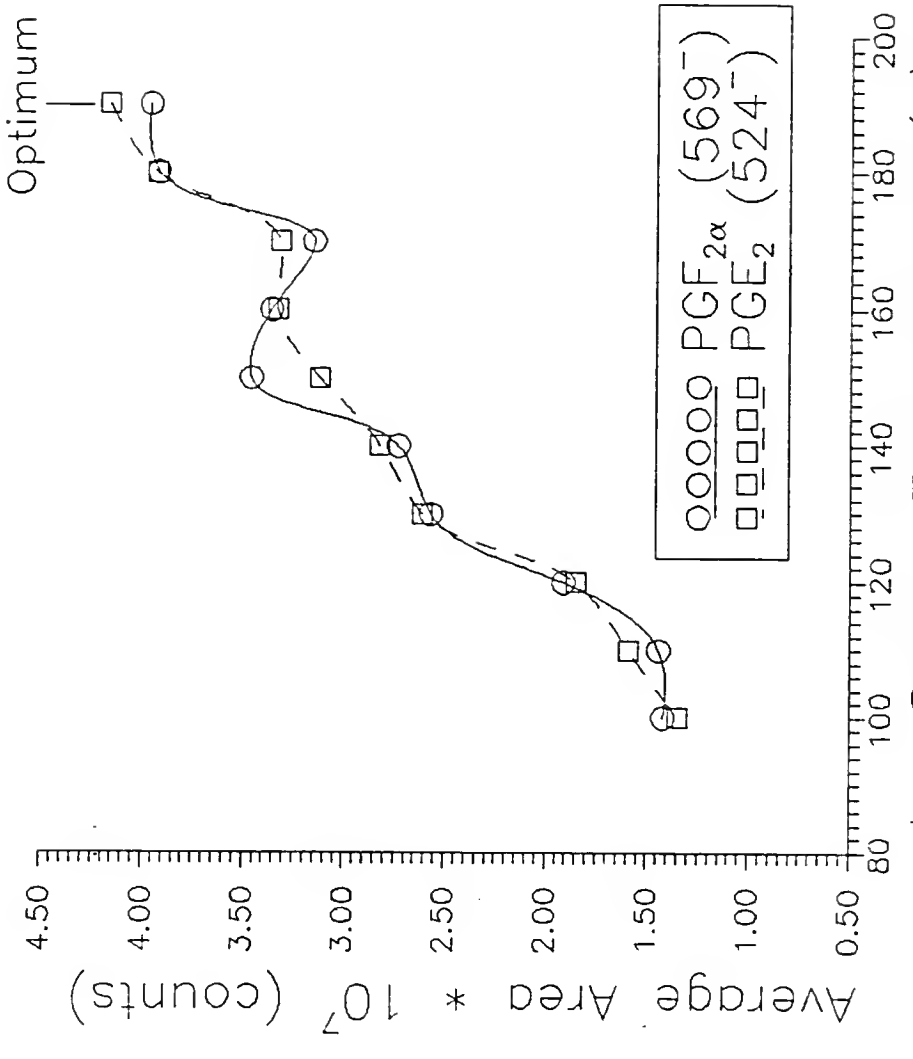


Figure 3-2: Ion source temperature study of the [M-PFB]$^-$ ion of PGE_2 and $PGF_{2\alpha}$.

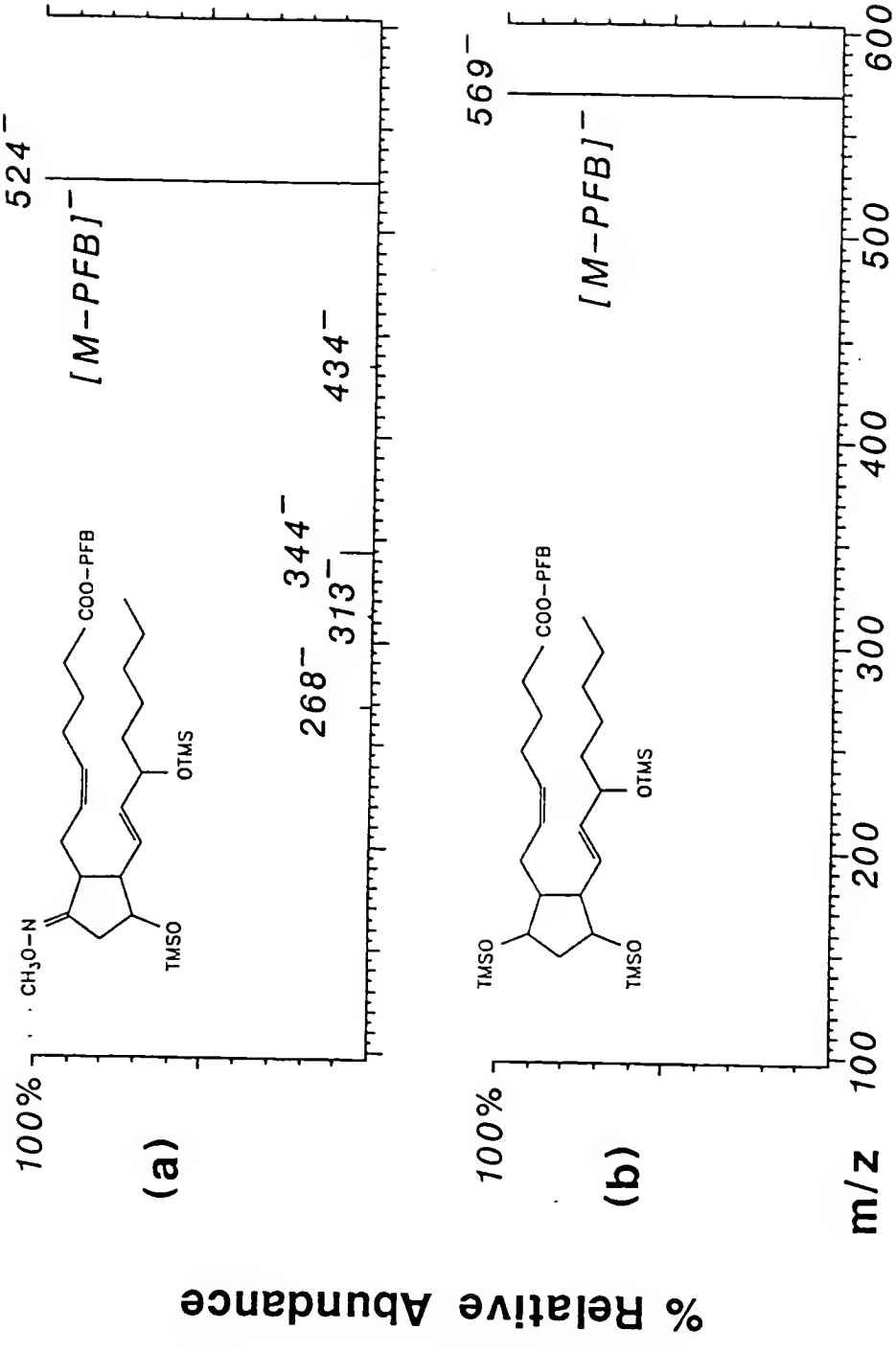


Figure 3-3: Electron-capture negative chemical ionization mass spectra of:
(a) PGE_2 MO/PFB/TMS derivative
(b) $PGF_{2\alpha}$ PFB/TMS derivative

prostaglandin analysis. One intense peak, the $[M-PFB]^-$ ion, dominates each mass spectrum. This ion, PGE_2 (524^-) and $PGF_{2\alpha}$ (569^-), can be utilized for SIM. Other low intensity fragment ions can be seen in the mass spectrum of PGE_2, corresponding to the loss of derivatives attached to PGE_2. In addition, no fragments of greater than 1% relative abundance are observed in the mass spectrum of $PGF_{2\alpha}$.

The M^- ion for both PGE_2 (m/z 705) and $PGF_{2\alpha}$ (m/z 750) is rarely present in the EC-NCI mass spectra, thus it must be less than 0.1% relative abundance. In addition, the PFB^- ion (m/z 181) occurs in the EC-NCI mass spectra of both PGs at less than 0.5% abundance.

Selected-Ion Monitoring Calibration Curve

A calibration curve for PGE_2 (524^-) is shown in Figure 3-4. This curve indicates the linearity and limit of detection for PGE_2 with SIM. Three one-microliter injections at nine different concentrations were performed. The limit of detection was calculated from the calibration data and corresponded to the amount of PGE_2 which could give a GC peak area three times greater than the average area obtained with a derivatized blank. The use of SIM with standard PGE_2 produced a limit of detection of approximately 94 fg (femtograms) and is indicated on the curve. The calibration curve showed good linearity above the limit of detection in the range of concentrations expected for endogenous PGE_2 in urine (100 to 400 pg/mL) (71,78). The linear dynamic range of the curve is from 500 fg to 1 ng (solid line) and the slope of the linear regression best fit line is 1.266 with a correlation coefficient of 0.9925. The non-linearity at the low end of the calibration curve may be due to adsorption on the

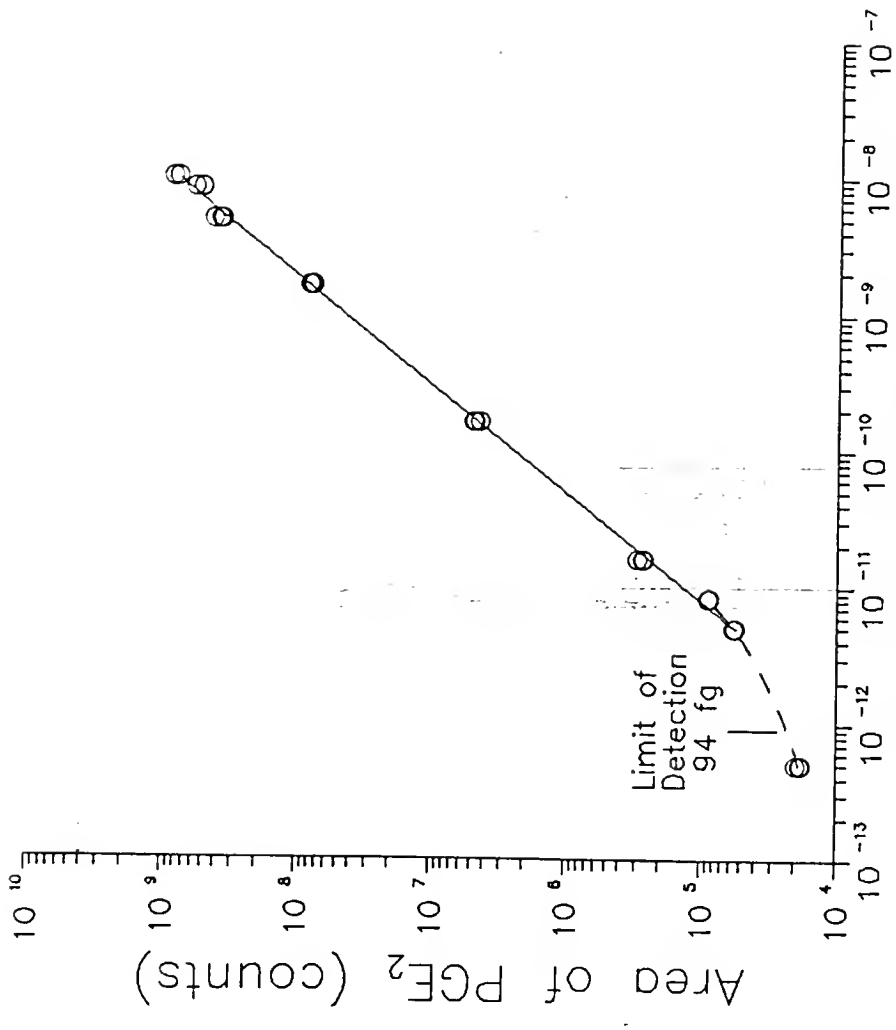


Figure 3-4: Selected-ion monitoring calibration curve for the $[M-PFB]^-$ ion of the MO/PFB/TMS derivative of PGE_2.

column, septum or injection port and subsequent adsorption by the next injection. Precision of the GC/MS method utilizing SIM was determined by performing ten one-microliter injections of a 50 pg/μL solution of PGE_2. The percent relative standard deviation (%RSD) of the ten injections was 5.5%. Calibration curves for $PGF_{2\alpha}$, PGD_2 and $DHKF_{2\alpha}$ were similar, with varying limits of detection, in the range of 50 to 200 fg.

The results for this study agree well with the literature. Reports have shown LODs using EI/MS at about 20 pg for the $M^{+\cdot}$ (6,17,48,62,101) and utilizing EC-NCI/MS about 100 fg (19,20,76,77) for the $[M\text{-}PFB]^-$ ion.

Tandem Mass Spectrometry (GC/MS/MS)

Monitoring the efficiencies of the collisionally activated dissociation processes can help in determining optimum MS/MS conditions for trace determination of PGs. These efficiencies are affected by collision energy and collision gas pressure. Either parameter can be varied to maximize the CAD efficiency for a particular parent ion. Increasing the collision energy allows for more energetic collisions, while increasing the collision gas pressure increases the number of collisions each ion experiences.

Collision Gas Pressure Studies

The collision gas pressure for three different gases (N_2, Ar, and Xe) was optimized to determine which collision gas and pressure were the most efficient for selected-reaction monitoring at the maximum available collision energy of 30 eV. Pressure-resolved breakdown curves for selected ions of the MO/PFB/TMS derivative of PGE_2 are shown in Figures

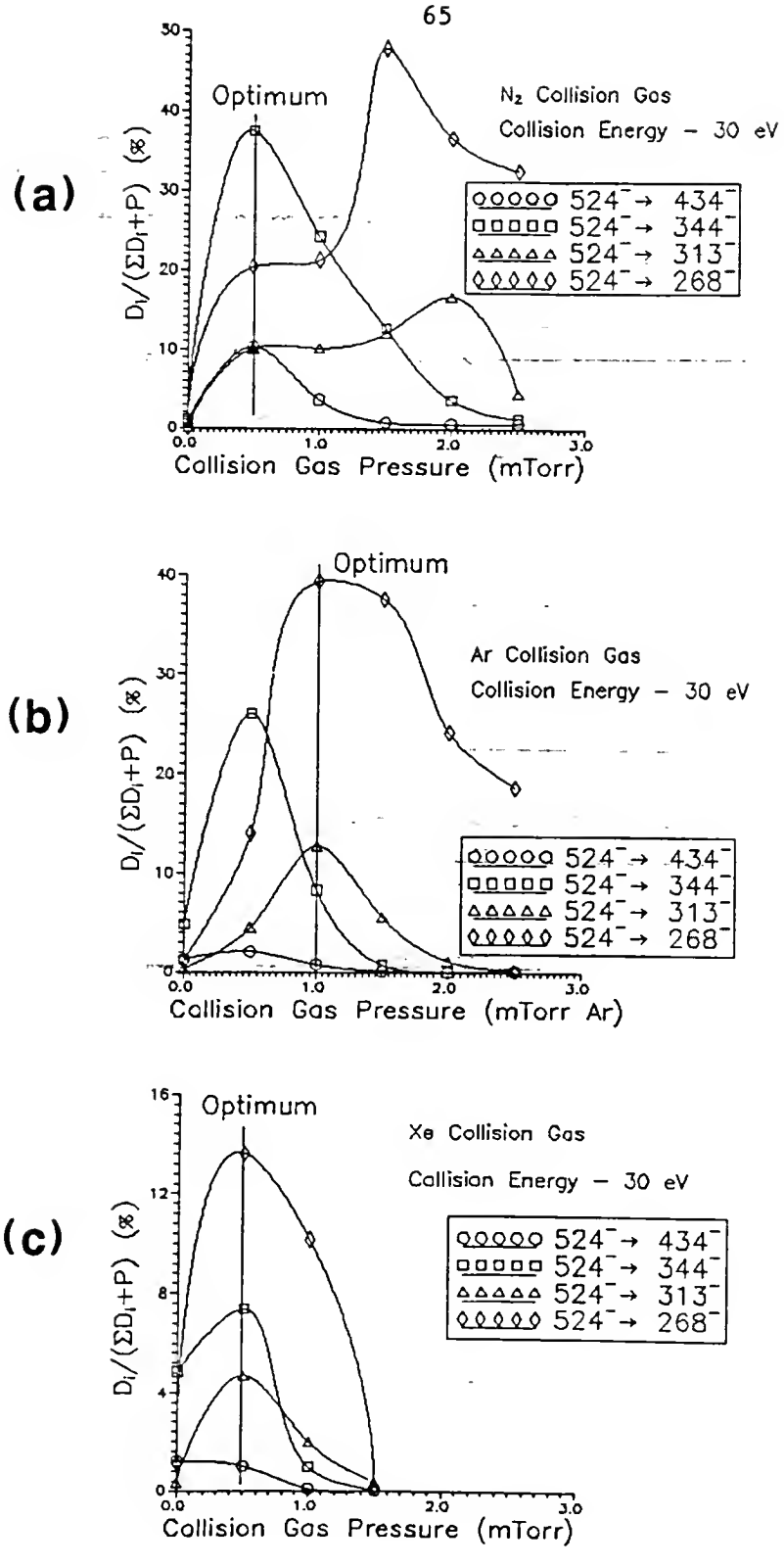


Figure 3-5: Pressure-resolved breakdown curve of the carboxylate anion of the MO/PFB/TMS derivative of PGE_2 with collision gas: (a) Nitrogen (b) Argon (c) Xenon

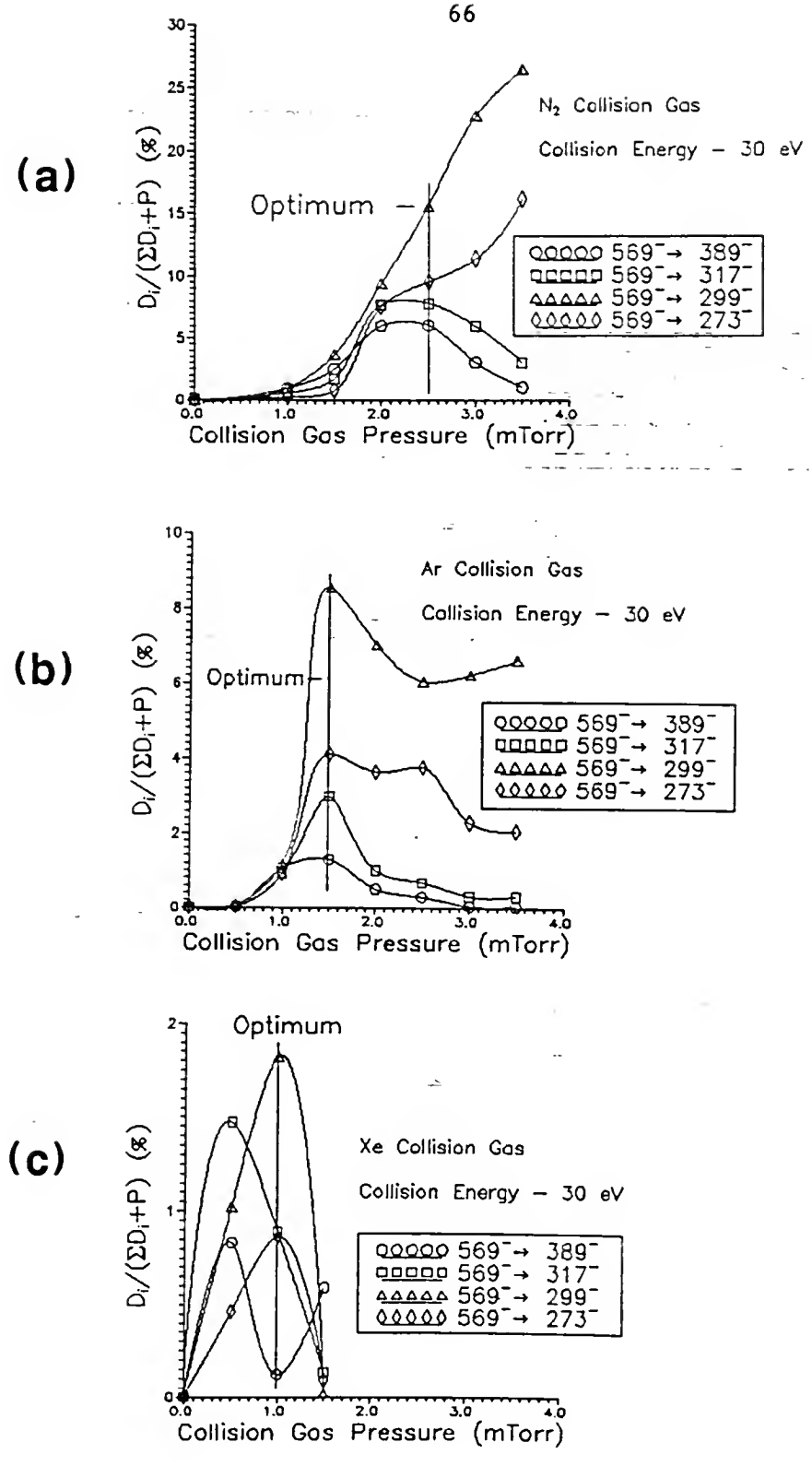


Figure 3-6: Pressure-resolved breakdown curve of the carboxylate anion of the PFB/TMS derivative of $PGF_{2\alpha}$ with collision gas: (a) Nitrogen (b) Argon (c) Xenon

3-5a, 3-5b and 3-5c and for the PFB/TMS derivative of $PGF_{2\alpha}$ in Figures 3-6a, 3-6b and 3-6c. The optimum collision gas pressure is indicated on each curve. This type of curve can be calculated by dividing the area of a selected daughter ion by the area of all the ions in the daughter spectrum ($D_i/[\Sigma D_i + P]$) at each pressure. The point which is chosen as the optimum is the pressure where one can obtain a qualitative daughter spectrum which is "rich" in structural information with a number of reasonably abundant daughter ions.

The optima indicated on Figures 3-5a, 3-5b and 3-5c occur at a collision pressure of 0.5, 1.0 and 0.5 mTorr for N_2, Ar, and Xe respectively. However, in the case of $PGF_{2\alpha}$, the optima occur at significantly higher pressures, 2.5, 1.5, and 1.0 mTorr for N_2, Ar, and Xe (Figures 3-6a, 3-6b and 3-6c). Comparing Figure 3-5a to 3-6a, the optimal use of nitrogen as a collision gas requires a pressure five times higher for $PGF_{2\alpha}$ than for PGE_2. This dramatic difference in optimum collision pressure exists between two structurally similar PGs.

The relative differences in the optimum collision gas pressure for $PGF_{2\alpha}$ with the various collision gases can be explained by the relative mass of the three different collision gases. The greater size of the argon and xenon gas molecules increases the energy deposited into the parent ion, therefore increasing the fragmentation efficiency. Therefore, the optimum collision pressure decreases with an increase in the mass of the gas molecules, because the abundance of the most prominent fragment ions occur at lower pressures.

Notice the significant differences in the maximum relative intensity of the daughter ions for the three collision gases. The curves with

nitrogen (Figures 3-5a and 3-6a) show a higher maximum relative intensity for the selected reactions listed. Figure 3-6c for $PGF_{2\alpha}$, utilizing xenon as the collision gas, is particularly interesting. The relative intensity of the daughter ions selected are 12 times lower than the intensity of the same daughter ions displayed in Figure 3-6a for nitrogen. In addition, the relative intensity of the daughter ions approach zero at higher collision gas pressures (> 1.0 mTorr). Thus, either the parent ion (carboxylate anion) is increasing at higher pressures or other daughter ions are more abundant with argon and xenon at these collision gas pressures. Clearly, for the CAD process, the parent ion decreases as the collision gas pressure is increased. Therefore, various daughter ions not listed in these figures must be prominent with argon and xenon at higher collision gas pressures.

This is apparent from examining Figure 3-7, which shows the pressure-resolved breakdown curves for different selected ions with the PFB/TMS derivative of $PGF_{2\alpha}$ with argon and xenon as collision gases. Comparing Figure 3-7a and Figure 3-7b to Figure 3-6a the relative intensity of the daughter ions for argon and xenon in this case are similar to that found for nitrogen. The most intense reaction in both cases in Figure 3-7 is the selected-reaction of $569^- \rightarrow 89^-$, corresponding to a back-bone fragmentation.

A general trend appears in all the figures for both PGE_2 and $PGF_{2\alpha}$. The loss of one and two HOTMS groups from the $[M\text{-}PFB]^-$ ion tend to maximize together at low collision gas pressures for the three collision gases. Subsequently, these selected-reactions gradually decrease towards zero at higher collision gas pressures. The four selected-reactions for PGE_2 in

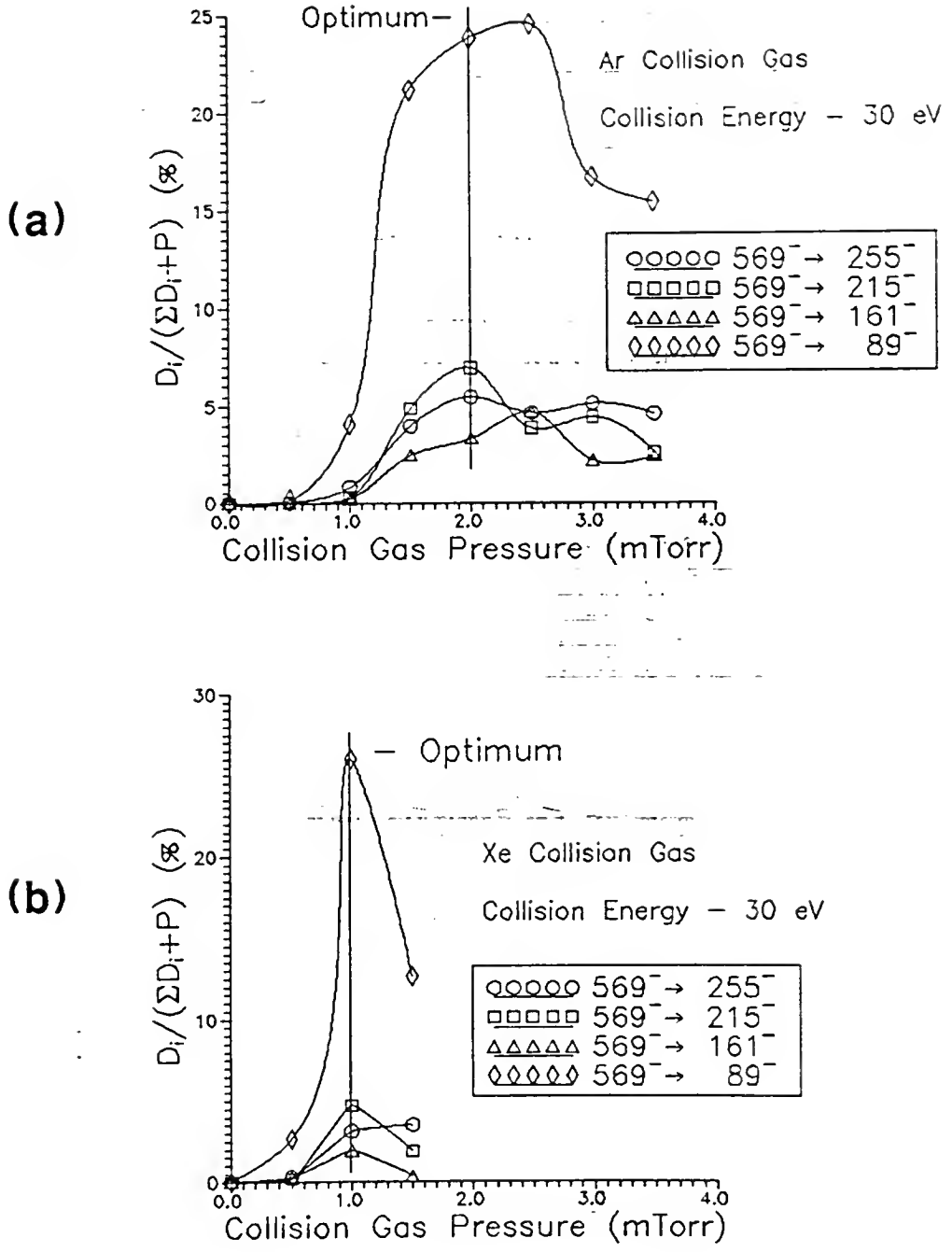


Figure 3-7: Pressure-resolved breakdown curve of the carboxylate anion of the PFB/TMS derivative of $PGF_{2\alpha}$ with collision gas: (a) Nitrogen (b) Argon

Figure 3-5 all increase at low collision gas pressures and then gradually decrease as higher collision gas pressures are employed. In the case of $PGF_{2\alpha}$, the additional loss of the third HOTMS group ($569^- \rightarrow 299^-$) gradually increases when nitrogen is utilized (Figure 3-6a) or levels off when argon (Figure 3-6b) is employed, as the collision gas pressure is continually increased. In Figure 3-7, the selected-reactions have relatively low intensities at low collision gas pressures (< 1.0 mTorr), but increase gradually and level off as the collision gas pressure is increased (> 1.0 mTorr).

Trace analysis by selected-reaction monitoring with MS/MS requires optimization of the absolute intensity of a single daughter ion of the selected parent ion. The curves in Figures 3-8 and 3-9 give an indication of the optimum reactions and collision gas pressures which should be selected for maximum SRM sensitivity for PGE_2 and $PGF_{2\alpha}$ with three different collision gases at maximum collision energy (30 eV). This type of curve is calculated by dividing the area of selected daughter ions, D_i, by the area of the incident parent ion, P_o (measured in a daughter spectrum without collision gas). The reaction with the highest CAD efficiency should be selected to yield the highest sensitivity for selected reaction monitoring (SRM) trace determination of PGs. For example, in the case of $PGF_{2\alpha}$, choice of the $569^- \rightarrow 299^-$ selected reaction with argon (Figure 3-9b) would be the optimum (overall efficiency of ~2%) at a collision pressure of 1.5 mtorr and collision energy of 30 eV. This reaction corresponds to the $[M\text{-}PFB]^- \rightarrow [(M\text{-}PFB) - 3(HOTMS)]^-$ for the derivatized carboxylate anion of $PGF_{2\alpha}$. Note that this overall CAD efficiency (~2%) is obtained at the optimum pressure for any of the three gases. The more

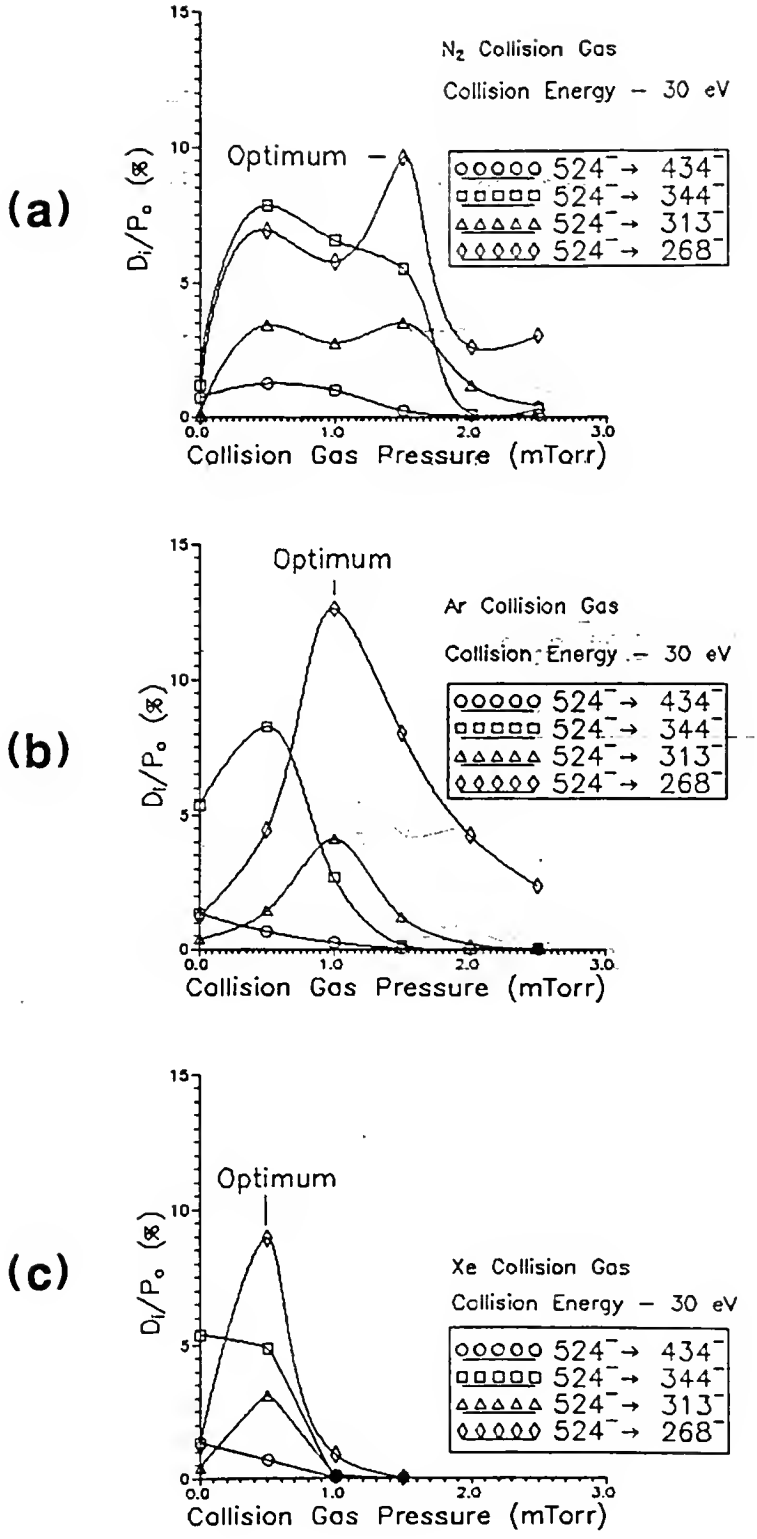


Figure 3-8: Overall CAD efficiency for the selected-reaction monitoring of the carboxylate anion of the MO/PFB/TMS derivative of PGE_2 with collision gas:
(a) Nitrogen (b) Argon (c) Xenon

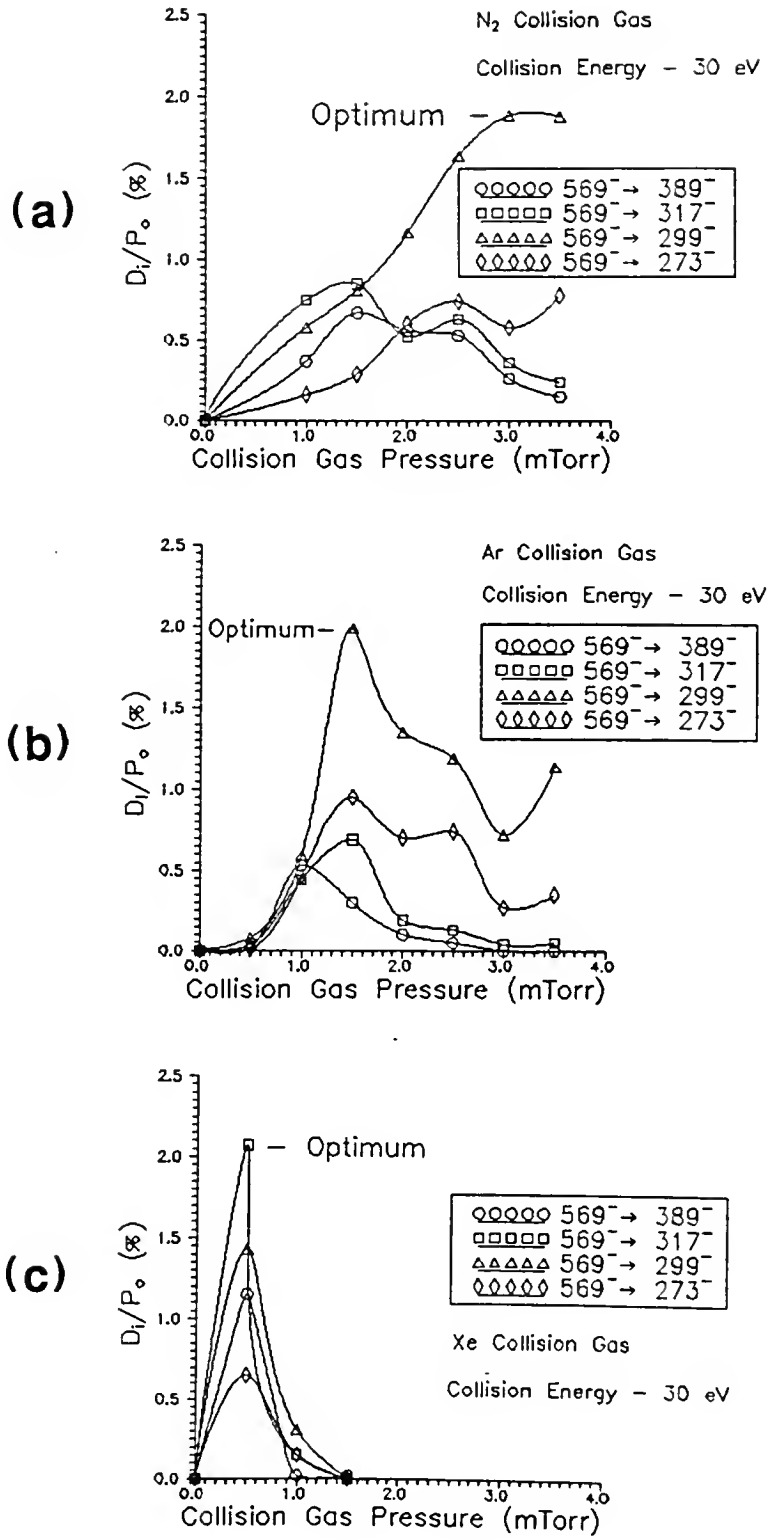


Figure 3-9: Overall CAD efficiency for the selected-reaction monitoring of the carboxylate anion of the PFB/TMS derivative of $PGF_{2\alpha}$ with collision gas: (a) Nitrogen (b) Argon (c) Xenon

massive the collision gas, the lower the optimum pressure. $PGF_{2\alpha}$ exhibits a slightly higher overall CAD efficiency (~2%) with xenon for the selected reaction of $569^- \rightarrow 317^-$ (Figure 3-9c). This reaction corresponds to the $[M\text{-}PFB]^- \rightarrow [(M\text{-}PFB) - 2(HOTMS) - (CH_3)_2Si{=}CH_2]^-$ for the derivatized carboxylate anion of $PGF_{2\alpha}$. This suggests that xenon would be the optimum CAD gas. However, xenon is quite expensive ($650/50 L of gas) and the gain in CAD efficiency is slight; thus, argon would be a more practical choice.

For PGE_2, the $[M\text{-}PFB]^- \rightarrow [(M\text{-}PFB) - 2(HOTMS) - CO_2 - HOCH_3]^-$ reaction with argon (Figure 3-8b) is optimum at a pressure 2 times lower than for $PGF_{2\alpha}$. Even more notable is that the optimum CAD efficiency (D_i/P_o) for PGE_2 (~10%) is significantly higher than that for $PGF_{2\alpha}$ (~2%) with all three gases employed. Note that on Figures 3-8b and 3-8c the lowest collision gas pressure been plotted is 0.2 mTorr. This is due to the fact that when the zero collision gas pressure data was obtained, the actual pressure of the collision cell was 0.2 mTorr. This indicates that residual collision gas was present in both these cases, thus allowing residual CAD to occur.

Collision Energy Study

Argon was chosen as the collision gas for the optimization of collision energy. The collision energy study for selected-reaction monitoring is shown in Figure 3-10. Data for SRM with the reactions and Argon pressure chosen as optimum in the collision gas pressure studies at 30 eV for PGE_2 and $PGF_{2\alpha}$ are plotted on the same graph. Three one-microliter injections of a 500 pg/μL solution of PGE_2 and $PGF_{2\alpha}$ were

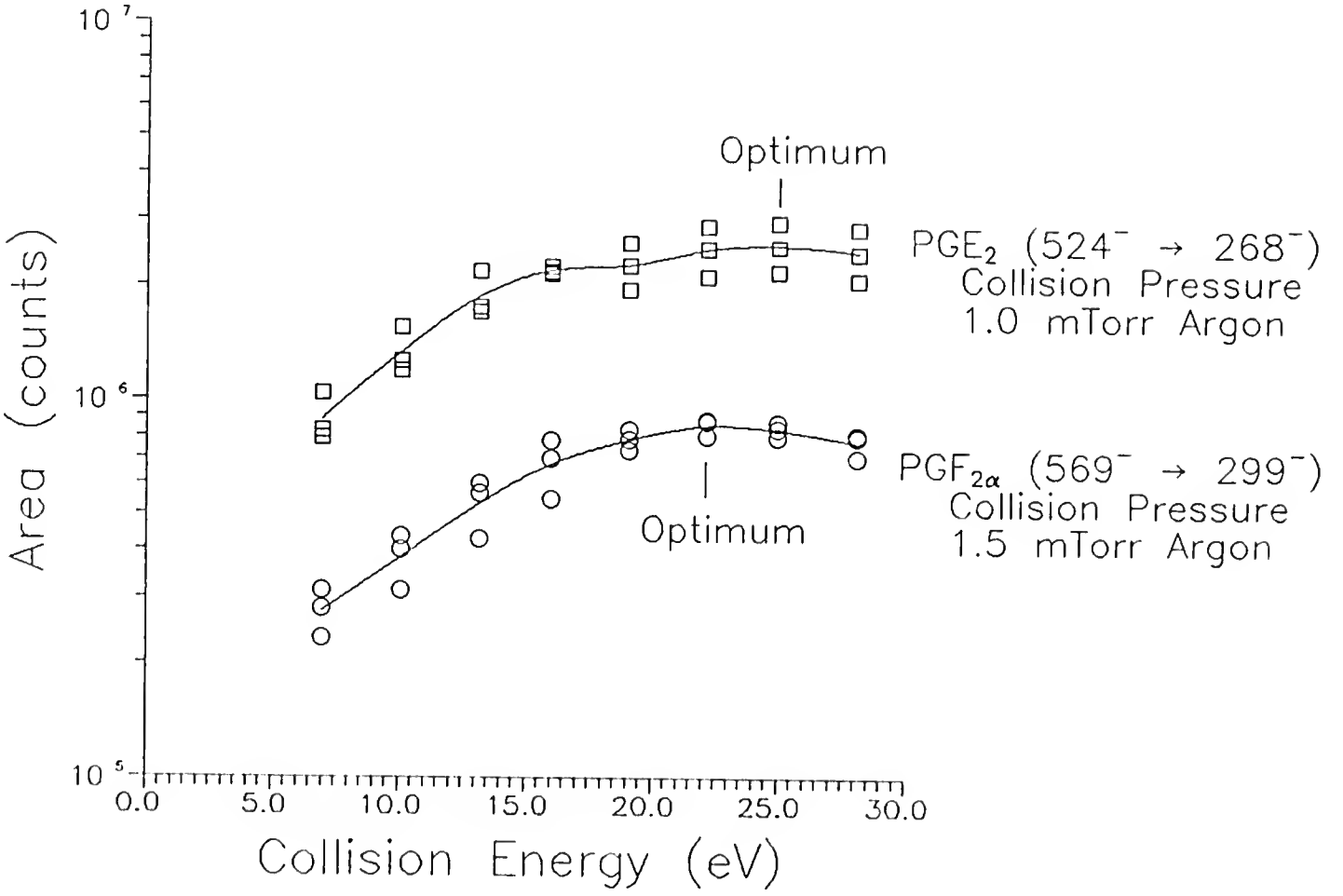


Figure 3-10: Collision energy study for the selected reactions of the MO/PFB/TMS derivative of PGE_2 and the PFB/TMS derivative of $PGF_{2\alpha}$.

performed at collision energies of 7 to 28 eV. Optima for the selected reactions of $524^- \rightarrow 268^-$ (PGE_2) and $569^- \rightarrow 299^-$ ($PGF_{2\alpha}$) are indicated on the graph. The optimum collision energy for PGE_2 (25 eV) is slightly higher than for $PGF_{2\alpha}$ (22 eV), probably due to the difference in the collision pressures employed for each selected reaction. These plots suggest that, once a particular collision gas, pressure, and selected reaction are chosen, variation of the collision energy has little effect.

Daughter Spectra of Standards

Figures 3-11a and 3-11b show daughter ion spectra for the carboxylate anions of the MO/PFB/TMS derivative of PGE_2 and the PFB/TMS derivative of $PGF_{2\alpha}$. The fragment ions labeled in the spectra are tabulated in Table 3-1 with possible assignments of the ion's identity. Most of the fragment ions observed in both daughter spectra are derivative-specific. These ions occur at m/z 434, 344 and 313 for PGE_2 and at m/z 479, 389, 317 and 299 for $PGF_{2\alpha}$. The most intense daughter ion from the fragmentation of the $[M-PFB]^-$ ion of PGE_2 is m/z 268 ion and corresponds to the loss of (2*HOTMS-CO_2-CH_3OH) from the parent ion of 524^-. In the daughter spectrum of $PGF_{2\alpha}$ the m/z 299 ion is the most intense and corresponds to the loss of three HOTMS groups from the parent ion of 569^-. The fragment ions which occur at lower masses are ions that correspond to backbone-specific fragments. This means these ions correspond to fragmentation of the carbon-hydrogen skeleton in both PGE_2 and $PGF_{2\alpha}$. These ions include m/z 240 and 214 for PGE_2 and m/z 255, 215, 201 and 161 for $PGF_{2\alpha}$.

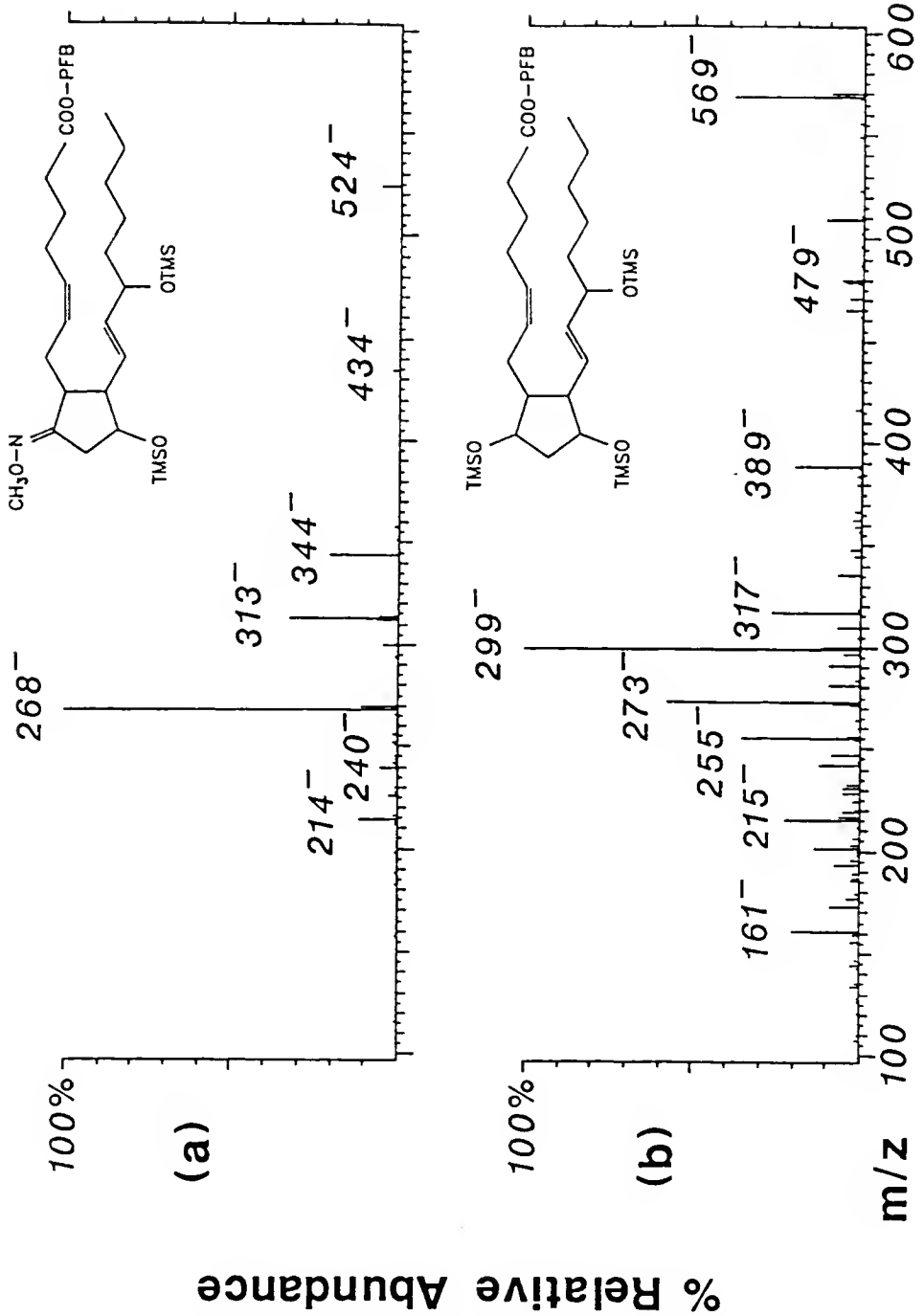


Figure 3-11: Daughter ion spectra of the $[M\text{-}PFB]^-$ ions (m/z 524 and 569) of:
(a) PGE_2 MO/PFB/TMS derivative at 1.0 mTorr argon and at 28.2 eV
(b) $PGF_{2\alpha}$ PFB/TMS derivative at 1.5 mTorr argon and at 28.2 eV

Table 3-1: Daughter Ions of $[M\text{-}PFB]^-$ (P^-) of PGE_2 MO/PFB/TMS and $PGF_{2\alpha}$ PFB/TMS

PGE_2 MO/PFB/TMS[a]

Ion Assignment	m/z	%RA[c]
P^-	524	5
$[P\text{-}HOTMS]^-$	434	2
$[P\text{-}2HOTMS]^-$	344	21
$[P\text{-}2HOTMS\text{-}OCH_3]^-$	313	32
$[P\text{-}2HOTMS\text{-}CO_2]^-$	300	4
$[P\text{-}2HOTMS\text{-}CO_2\text{-}CH_2O]^-$	270	11
$[P\text{-}2HOTMS\text{-}CO_2\text{-}CH_3OH]^-$	268	100
$[P\text{-}2HOTMS\text{-}CO_2\text{-}CH_3OH\text{-}C_2H_4]^-$	240	5
$[P\text{-}2HOTMS\text{-}CO_2\text{-}CH_3OH\text{-}C_4H_6]^-$	214	12

$PGF_{2\alpha}$ PFB/TMS[b]

Ion Assignment	m/z	%RA
P^-	569	36
$[P\text{-}HOTMS]^-$	479	4
$[P\text{-}2HOTMS]^-$	389	18
$[P\text{-}2HOTMS\text{-}(CH_3)_2Si{=}CH_2]^-$	317	24
$[P\text{-}3HOTMS]^-$	299	100
$[P\text{-}2HOTMS\text{-}CO_2\text{-}(CH_3)_2Si{=}CH_2]^-$	273	56
$[P\text{-}3HOTMS\text{-}CO_2]^-$	255	36
$[P\text{-}3HOTMS\text{-}CO_2\text{-}C_3H_4]^-$	215	21
$[P\text{-}3HOTMS\text{-}CO_2\text{-}C_4H_6]^-$	201	12
$[P\text{-}3HOTMS\text{-}CO_2\text{-}C_7H_{10}]^-$	161	18

[a] At a collision gas pressure of 1.0 mTorr argon and collision energy of 28.0 eV.

[b] At a collision gas pressure of 1.5 mTorr argon and collision energy of 28.0 eV.

[c] % Relative Abundance

Considerations for choice of a particular selected reaction for monitoring has been discussed recently by Strife (77). This report along with other studies have shown that backbone-specific fragmentation confers superior selectivity over derivative-specific fragmentation in the analysis of biological samples. For example, when SRM is based on a derivative-specific fragmentation, any component in a SIM chromatogram that is derivatized has an enhanced probability of appearing in a SRM chromatogram. However, the backbone-specific fragmentation has a lower relative daughter ion intensity in EC-NCI/GC/MS/MS than for derivative-specific fragmentation. Therefore, if the backbone-specific fragmentation was chosen for SRM analysis the sensitivity would be lower than that for the derivative-specific fragmentation found in Figure 3-8 and Figure 3-9 for PGE_2 and $PGF_{2\alpha}$.

Selected-Reaction Monitoring Calibration Curve

A selected-reaction monitoring (SRM) calibration curve for PGE_2 ($524^- \rightarrow 268^-$) is shown in Figure 3-12. This curve indicates the linearity and limit of detection for PGE_2 with SRM. Three one-microliter injections at seven different concentrations were performed. The limit of detection was calculated from the calibration data, the corresponding amount of PGE_2 which gives a GC peak area three times greater than the average area obtained with a derivatized blank. The use of SRM with standard PGE_2 produced a limit of detection of approximately 14 pg, as indicated on the curve. The calibration curve showed good linearity above the limit of detection in the range of concentrations expected for endogenous PGE_2 in urine (100 to 400 pg/mL) (71,78). The linear dynamic range of the curve

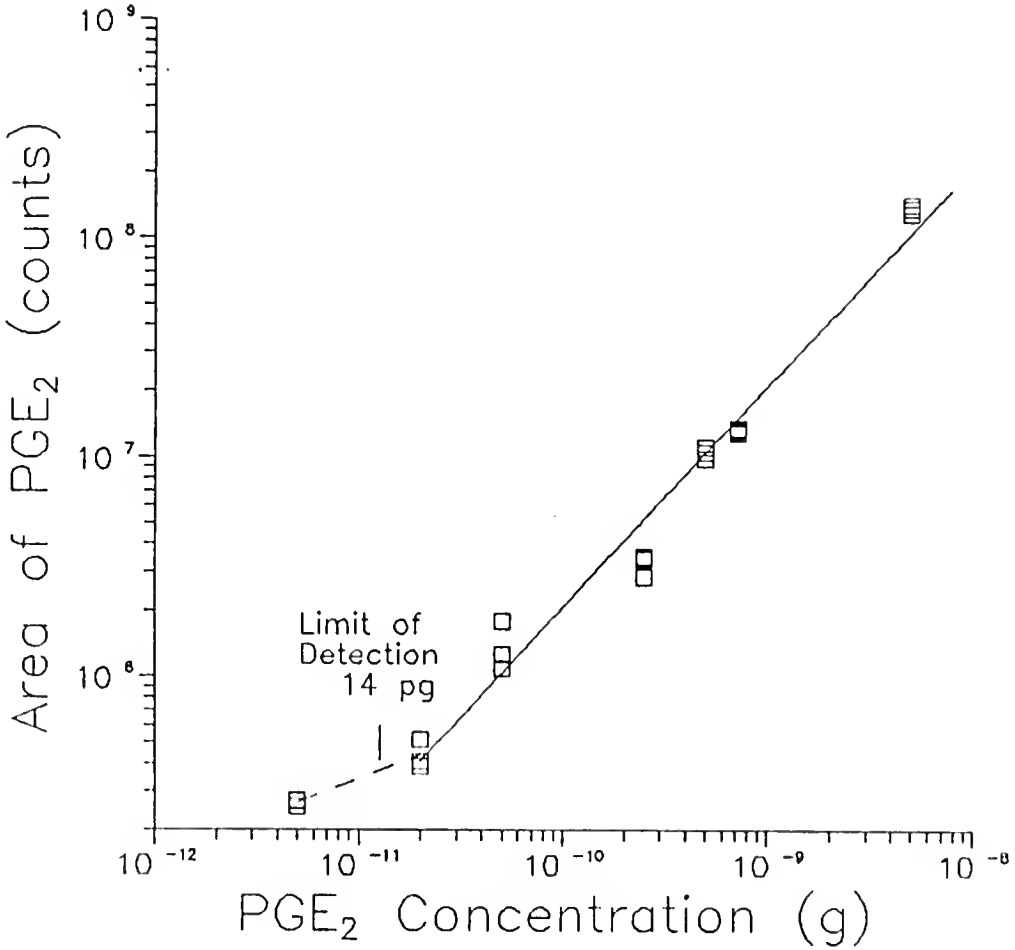


Figure 3-12: Selected-reaction monitoring calibration curve for the $524^- \rightarrow 268^-$ reaction of the MO/PFB/TMS derivative of PGE_2.

is from 20 pg to 5 ng (solid line) and the slope of the linear regression best fit line is 1.007 with a correlation coefficient of 0.9989. Precision of the GC/MS/MS method utilizing SRM was determined by performing ten one microliter injections of a 500 pg/μL solution of PGE_2. The percent relative standard deviation (%RSD) of the ten injections was 3.9%. Calibration curves for $PGF_{2\alpha}$, PGD_2 and $DHKF_{2\alpha}$ were similar, with varying limits of detection in the range of 5 to 30 pg. The results for this study agree well with literature reports, which have shown LODs utilizing EC-NCI/MS/MS of about 1 to 20 pg (19,20,76,77).

Conclusions

The optimum conditions for GC/MS electron-capture negative chemical ionization (EC-NCI) with SIM and GC/MS/MS with SRM are summarized in Table 3-2. The optimum collision gas pressure for both qualitative and quantitative (SRM) analysis of PGE_2 are lower than the optima found for $PGF_{2\alpha}$. The dramatically lower CAD efficiency for the carboxylate anion of $PGF_{2\alpha}$ (Figure 3-8b) compared to that of PGE_2 (Figure 3-7b) clearly indicates its greater stability under CAD conditions.

This study demonstrates the need for evaluating the CAD efficiency in the trace analysis of PGs. Optimization of both collision energy and collision gas pressure is essential in obtaining an accurate qualitative daughter spectrum "rich" in structural information. The CAD reaction with the highest CAD efficiency should be selected to yield the sensitivity for SRM determination of PGs.

Examining the calibration curves (Figures 3-4 and 3-11) differences between SIM and SRM are noted. Sensitivity is greater with SIM than with

Table 3-2: Otimum Conditions for Electron-Capture Negative Chemical Ionization Mass Spectrometry and Tandem Mass Spectrometry

Electron-Capture Negative Chemcial Ionization

Pararmeter	PGE_2	$PGF_{2\alpha}$
Ion Source Pressure	0.50 Torr	0.50 Torr
Ion Source Temperature	190°C	190°C

Tandem Mass Spectrometry

Qualitative Daughter Spectrum

Parameter	PGE_2	$PGF_{2\alpha}$
Collision Gas	Argon	Argon
Collision Gas Pressure	1.0 mTorr	1.5 mTorr
Collision Energy	28.1 eV	28.1 eV

Quantitative Selected-Reaction Monitoring

Parameter	PGE_2	$PGF_{2\alpha}$
Collision Gas	Argon	Argon
Selected Reaction	$524^- \rightarrow 268^-$	$569^- \rightarrow 299^-$
Collision Gas Pressure	1.0 mTorr	1.5 mTorr
Collision Energy	25.0 eV	22.2 eV

SRM. The limit of detection for SIM (94 fg) is slightly more than 2 orders of magnitude lower than for SRM (14 pg). Comparing the relative peak areas of SIM and SRM at 20 pg the SIM peak area is approximately 10 times higher than the peak area of 20 pg with SRM. In addition, at higher levels of PGE_2, 500 pg, the SIM peak area is approximately 40 times higher than the SRM peak area. The lower sensitivity of SRM is expected due to the limited efficiency of the CAD conversion of the parent ion to the daughter ion (approximately 12% for $524^- \rightarrow 268^-$) of interest, as well as transmission losses inherent in adding a second stage of mass analysis (typically 10 times). However, the selectivity gained by the parent-daughter reaction should reduce the chemical noise, in a sample matrix, to a greater extent than the analytical signal, thus, compensating for the lost sensitivity.

CHAPTER 4

DIFFERENCES IN THE COLLISIONALLY ACTIVATED DISSOCIATION OF STRUCTURALLY SIMILAR PROSTAGLANDINS

Introduction

The ions formed by electron ionization (EI) of the methyl ester/methoxime/trimethyl silyl ether derivatives of prostaglandins (PGs) show considerable fragmentation in the collisionally activated dissociation (CAD) process (74,75). However, the carboxylate anions of certain PGs produced by EC-NCI have been reported to be extremely stable when subjected to CAD (76).

It has been observed that the carboxylate anions of certain PGs exhibit little fragmentation even at high collision energies (>20 eV) and pressures (1.5 mTorr N_2). Subtle differences among the structures of prostaglandins E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$), D_2 (PGD_2) and 13,14-dihydro-15-keto $F_{2\alpha}$ ($DHKF_{2\alpha}$) (Figure 4-1) yield enormous differences in CAD efficiency. The CAD efficiency for the $[MO/TMS\text{-}PFB]^-$, $[M\text{-}PFB]^-$ and $[M\text{-}H]^-$ carboxylate anions is significantly different for closely related PGs. The low fragmentation and CAD efficiencies of the carboxylate anions of $PGF_{2\alpha}$ and $DHKF_{2\alpha}$ compared to those of PGE_2 and PGD_2 clearly indicate the greater stability of these species. In this chapter these differences are evaluated and explained in relation to the structural differences between the carboxylate anions for the PGs.

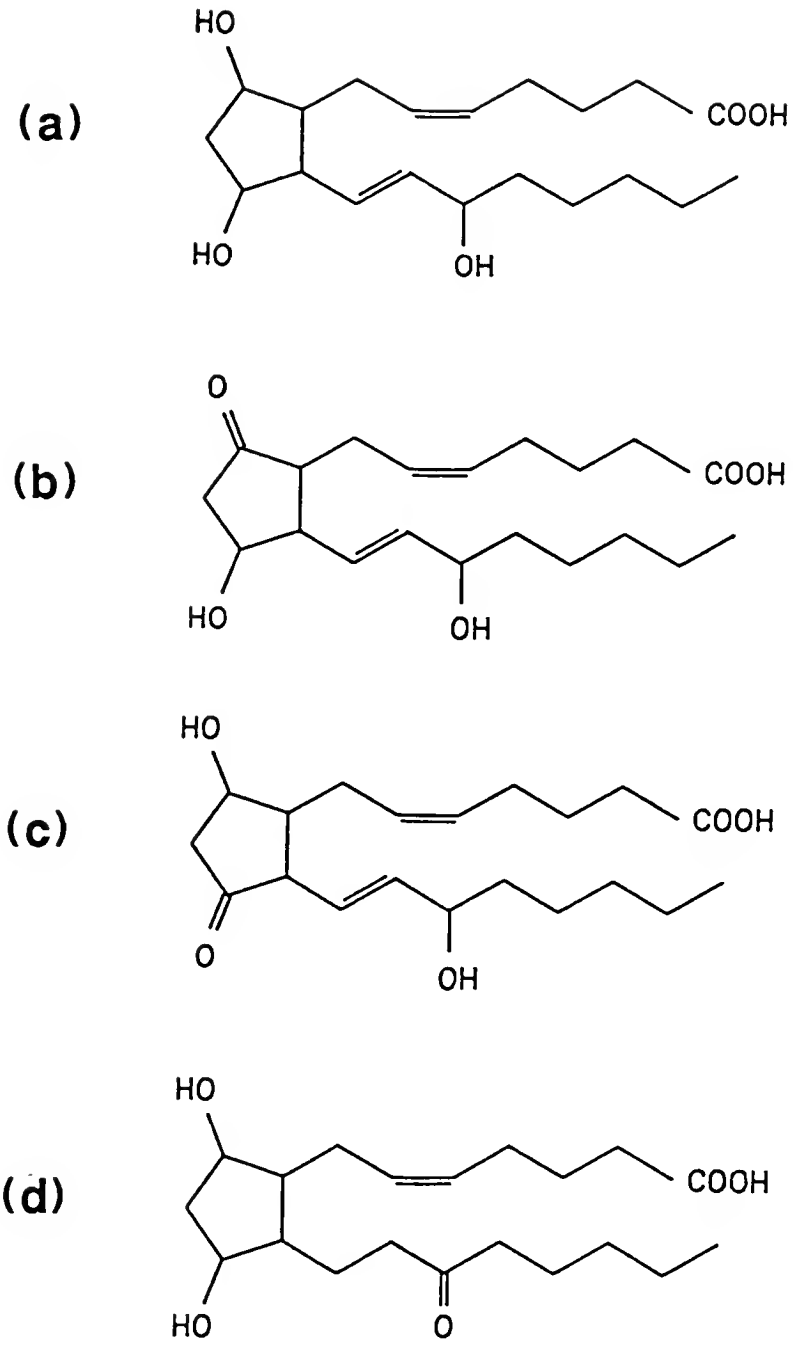


Figure 4-1: Structures of the four prostaglandins studied:
(a) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (b) Prostaglandin E_2 (PGE_2)
(c) Prostaglandin D_2 (PGD_2) (d) 13,14-dihydro-15-keto $F_{2\alpha}$ ($DHKF_{2\alpha}$)

Experimental

Prostaglandins and Reagents

The prostaglandins E_2, $F_{2\alpha}$, D_2 and 13,14-dihydro-15-keto $F_{2\alpha}$, as well as O-methylhydroxylamine hydrochloride, N,N-diisopropylethyl-amine, pyridine, and acetonitrile (analytical grade) were all purchased from Sigma Chemical Co.. Pentafluorobenzylbromide (PFBBr) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Pierce Chemical Co.. The methane (>99%) used as the chemical ionization reagent gas was purchased from Matheson Gas Products, Inc.. Helium used as GC carrier gas and nitrogen used as CAD collision gas were commercial grade, with their purity checked by mass spectrometry.

Derivatization

The methoxime/pentafluorobenzyl ester/trimethylsilyl (MO/PFB/TMS) derivatives (Figure 4-2) formed for the GC/MS/MS studies were prepared according to the method in chapter 2. The trimethylsilyl derivative was formed by adding 100 μL of BSTFA and allowed to stand overnight at room temperature. Dilutions were made from this solution so that a 500 pg/μL solution of each PG was used for injections. The solids probe/MS/MS studies were performed either by analyzing the standards without derivatization or as the PFB derivative, using only the PFBBr esterification step above.

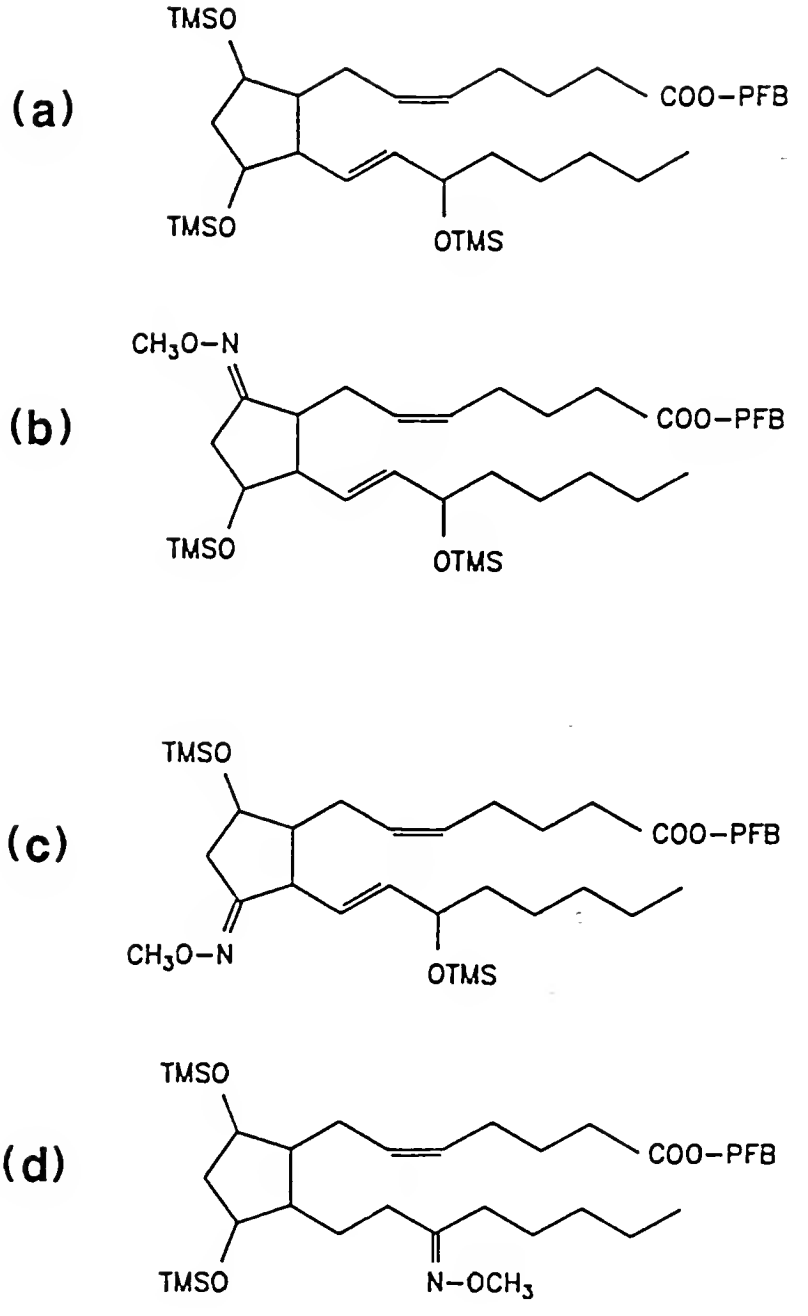


Figure 4-2: Structures of the methoxime-pentafluorobenzyl-trimethylsilyl (MO/PFB/TMS) derivatives of the four prostaglandins:
(a) $PGF_{2\alpha}$ (b) PGE_2 (c) PGD_2 (d) $DHKF_{2\alpha}$

Instrumental Conditions

GC was carried out on a short J&W Scientific (Folsom, CA) DB-1 (3 m long, 0.25 mm i.d., 0.25 μm film thickness) capillary column in the splitless mode with helium carrier gas at an inlet pressure of 4-6 psi. The initial temperature of 200°C was held for 30 s, then increased at 20°C/min to 260°C. The injector temperature was 300°C. One-microliter injections of a 500 pg/μL solution of each PG were made in triplicate at each condition for the GC/MS/MS studies.

The solids probe was used as the means for sample introduction to study the PFB ester derivatives and the free (underivatized) PG standards. The initial temperature was 50°C and increased at 20°C/min to 300°. Triplicate samples were analyzed for each derivatization procedure at each condition for the MS/MS studies. Sample size was one microgram of the underivatized PGs or 1 ng of the PFB ester derivatives.

A Finnigan MAT TSQ45 gas chromatograph/triple quadrupole mass spectrometer was employed. Mass spectrometry conditions were: interface and transfer line temperature 300°C, ionizer temperature 190°C, electron energy 100 eV and emission current 0.3 mA. Electron-capture negative chemical ionization (EC-NCI) was carried out with methane at an ionizer pressure of 0.45 torr.

In the MS/MS experiments, nitrogen collision gas pressure and collision energy were varied depending on each experiment. The $[MO/TMS\text{-}PFB]^-$, $[M\text{-}PFB]^-$ carboxylate anions were selected in the first quadrupole (Q1) region and passed into the collision cell (Q2). In this region these ions underwent CAD to form characteristic fragments which were then mass analyzed in the third quadrupole (Q3) region. A full daughter spectrum

was acquired over the mass range of 55-600 amu. The maximum collision energy possible on the TSQ 45 is 30 eV.

The peak areas in the daughter spectra of selected daughter ions and the parent ion remaining after CAD were calculated by the INCOS computer system for each GC and solids probe sample. A baseline was chosen visually and the calculated areas were used for determining CAD efficiencies.

Efficiency Calculations

The abundance of the daughter ions relative to that of the remaining parent carboxylate anion in the daughter spectrum can be controlled by varying the CAD energy or pressure; these parameters also affect sensitivity due to scattering losses. The processes of fragmentation and scattering can be monitored by evaluating the fragmentation (E_F), collection (E_C), and overall CAD (E_{CAD}) efficiencies given by the following equations (110):

$$E_F = \frac{\Sigma D_i}{P + \Sigma D_i} = \text{fraction of ions present following CAD which are daughter ions}$$

$$E_C = \frac{P + \Sigma D_i}{P_0} = \text{fraction of initial parent ions that is collected following CAD as either parent or daughter ions}$$

$$E_{CAD} = \frac{\Sigma D_i}{P_0} = \text{fraction of initial parent ion that is converted to collectable daughter ions}$$

where P_0, P, and D_i are the intensities of the parent ion prior to CAD, the parent ion remaining after CAD, and a daughter ion resulting from CAD, respectively. Note that $E_{CAD} = E_F \times E_C$.

As was stated earlier in chapter 3, the above efficiencies are affected by collision energy and collision gas pressure. Either parameter can be varied to maximize the CAD efficiency for a particular parent ion. Increasing the collision energy allows for more energetic collisions, while increasing the collision gas pressure increases the number of collisions each ion experiences. Either approach increases the amount of energy deposited into the parent ion, and thereby increases the fragmentation efficiency. However, an increase in collision energy or pressure will produce an increase in scattering losses (or possibly other loss mechanisms such as neutralization by charge exchange) and thereby decrease collection efficiency. The overall CAD efficiency, as the product of fragmentation and collection efficiency, will typically first increase then level off and even decrease as the collision energy or pressure are increased. Systematic variation of each parameter would provide a three-dimensional plot of efficiency vs. energy vs. pressure. Practically, such studies involve two-dimensional slices through this three-dimensional surface, varying one parameter while keeping the other constant.

Collision Energy Study of the [MO/TMS-PFB]$^-$ Carboxylate Anions

In light of the dramatic differences observed in Chapter 3 of the CAD efficiencies of the carboxylate anions of the fully derivatized $PGF_{2\alpha}$ and PGE_2 (differing structurally only in the presence of a carbonyl (=O)

group at C-9 derivatized to a methoximine, in PGE_2, rather than a hydroxyl (OH) group at the C-9 position, derivatized to a trimethylsilyl group, in $PGF_{2\alpha}$) two PGs similar to these were studied. The PGD_2 and PGE_2 isomers vary only by the interchange of the hydroxyl (OH) and carbonyl (=O) groups on C-9 and C-11. $DHKF_{2\alpha}$ differs from $PGF_{2\alpha}$ by exchange of a carbonyl (=O) group (derivatized to a methoximine) for the hydroxyl (OH) group (derivatized to a trimethylsilyl) at C-15.

Figure 4-3 and Figure 4-4 presents curves for fragmentation, collection and overall CAD efficiencies versus collision energy for the carboxylate anions of PGE_2, $PGF_{2\alpha}$, PGD_2 and $DHKF_{2\alpha}$. These curves show the effects of varying the collision energy at two different collision gas pressures. In Figures 4-3a, 4-3b and 4-3c, the collision pressure has been established at 1.2 mTorr N_2, a value which is typically optimum for many MS/MS analyses. The fragmentation efficiency curve (Figure 4-3a) indicates the dramatic differences in stability of the carboxylate anions of the four PGs. At a collision energy of 30 eV the fragmentation efficiencies ranges from a typical 80% down to only 2%. The collision pressure must be increased to produce more efficient fragmentation. The collection efficiency (Figure 4-3b) for the four PGs are similar. The notable exception is PGD_2, which has an unusually high collection efficiency at collision energies of 10-25 eV. This explains the difference noted between the fragmentation efficiency of PGE_2 and PGD_2 compared to their overall CAD efficiency.

Figures 4-4a, 4-4b and 4-4c show the overall CAD, collection and fragmentation efficiencies at a collision gas pressure 2.5 times higher. The fragmentation efficiency (Figure 4-4a) as well as the overall CAD

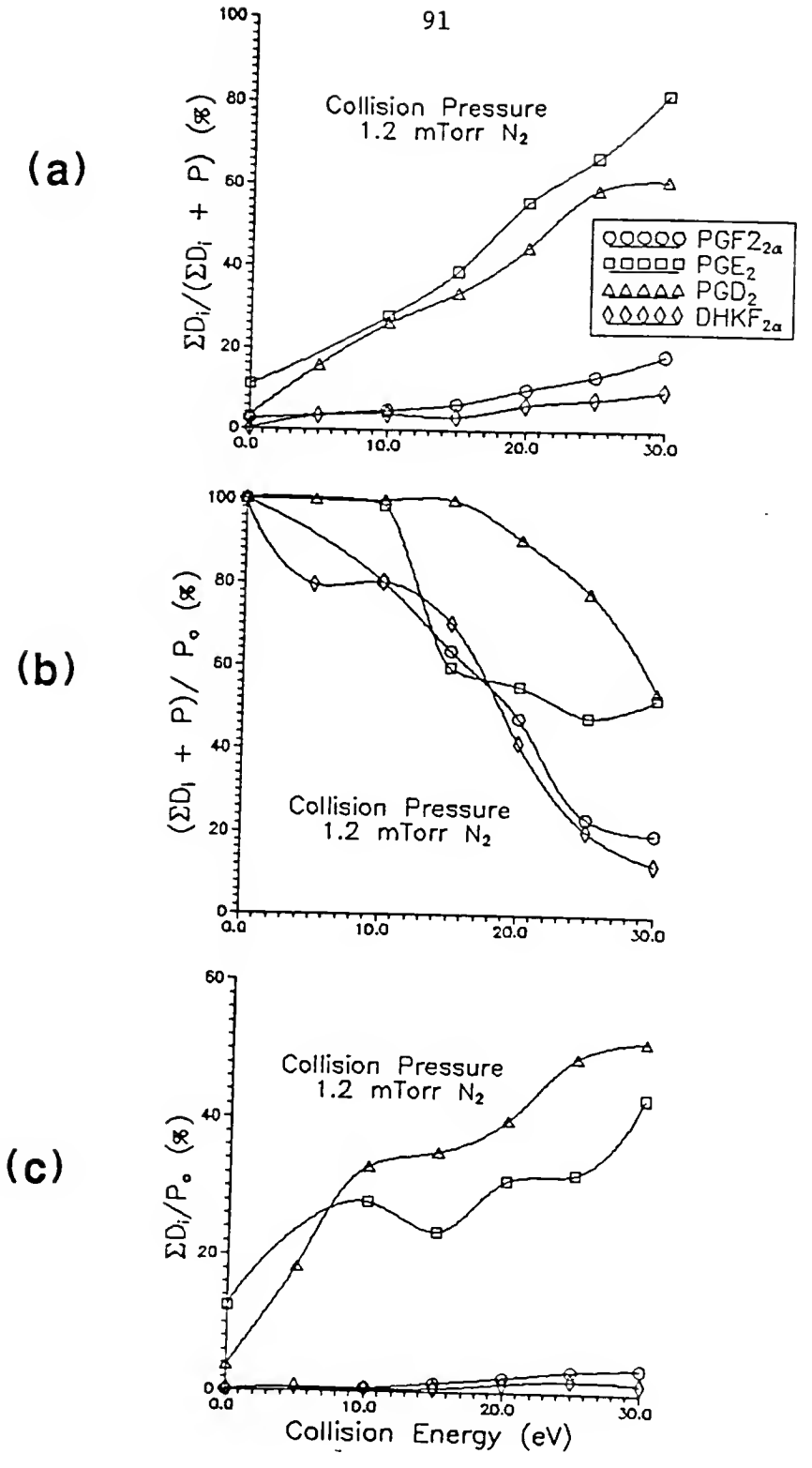


Figure 4-3: CAD efficiency of the [MO/TMS - PFB]$^-$ carboxylate anions versus collision energy at 1.2 mTorr N_2:
(a) Fragmentation
(b) Collection
(c) Overall CAD

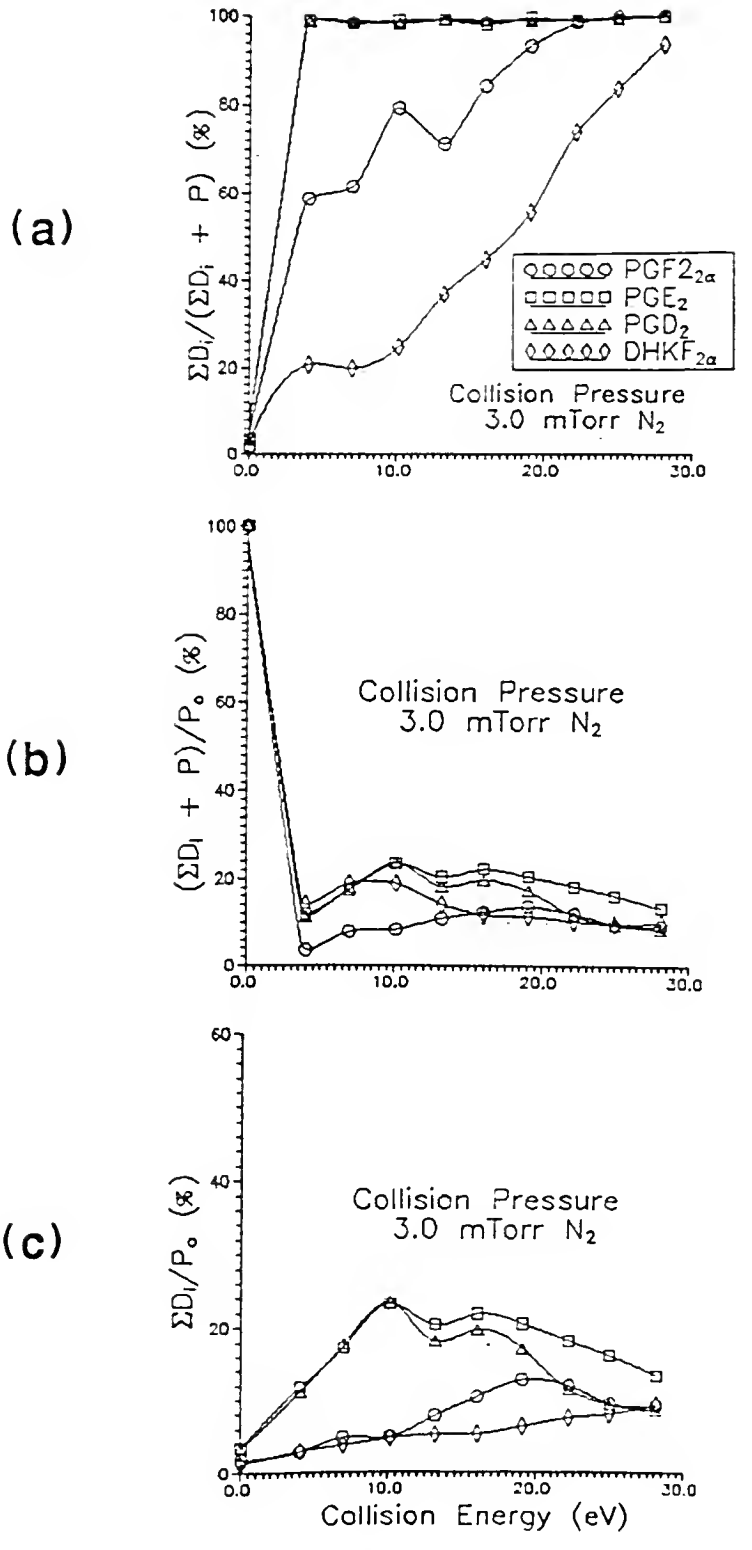


Figure 4-4: CAD efficiency of the [MO/TMS - PFB]$^-$ carboxylate anions versus collision energy at 3.0 mTorr N_2:
(a) Fragmentation
(b) Collection
(c) Overall CAD

efficiency (Figure 4-4c) curves demonstrate the differences in the stability of the carboxylate anions during the CAD process. At these high collision gas pressures, even at a collision energy as low as 4.0 eV, little or no parent ion is left for PGE_2 and PGD_2, yielding a fragmentation efficiency of nearly 100% (Figure 4-4a). The inefficient fragmentation (noted in Figure 4-3a) for $PGF_{2\alpha}$ and $DHKF_{2\alpha}$ is still observed. Note that, at the higher collision gas pressure, scattering losses become significant, especially at higher collision energies, yielding an optimum collision energy below the maximum available 30 eV.

Collision Pressure Study of the $[MO/TMS\text{-}PFB]^-$ Carboxylate Anions

The fragmentation efficiencies (E_F) for the carboxylate anions of the four PGs as a function of collision gas pressure at the maximum available collision energy (30 eV) are shown in Figure 4-5a. At low collision gas pressures, PGE_2 has a fragmentation efficiency 20 times greater than that for $PGF_{2\alpha}$ and $DHKF_{2\alpha}$. There are no dramatic differences in the collection efficiency (Figure 4-5b) of the four PGs, although at low collision gas pressures (< 0.5 mTorr N_2), $DHKF_{2\alpha}$ has the highest E_C, but at high collision gas pressures the lowest E_C. Since the mass of the carboxylate anions are approximately the same, the increased loss of ions is probably not due to scattering, but rather due to neutralization of the anion by electron detachment. The overall CAD efficiencies (Figure 4-5c) for the carboxylate anions of the four PGs demonstrate the differences in their behavior; PGE_2 has a maximum E_{CAD} of 35%, which is 2 times greater than that for PGD_2, 5 times better than $PGF_{2\alpha}$ and 20 times greater than $DHKF_{2\alpha}$.

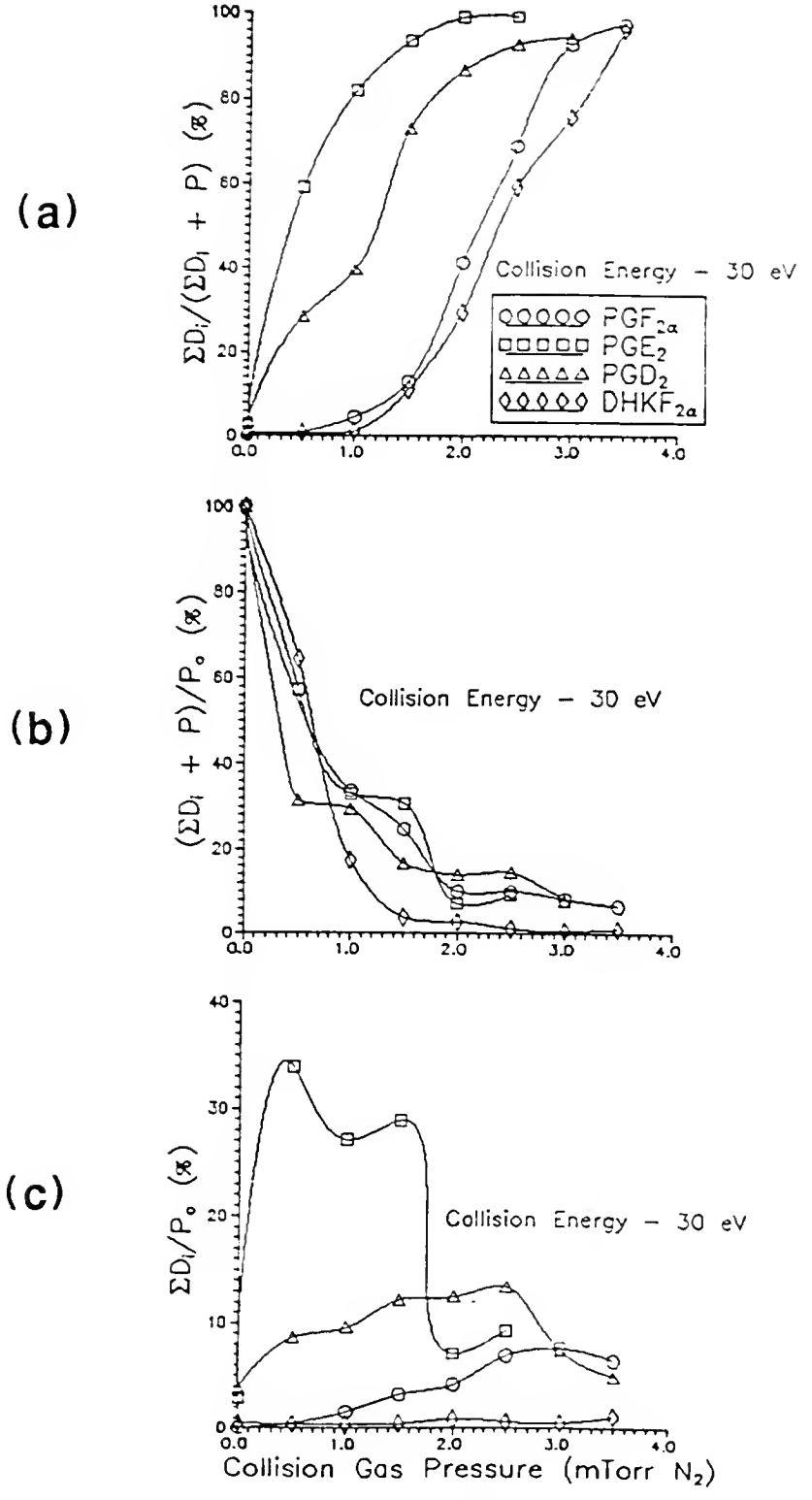


Figure 4-5: CAD efficiency of the [MO/TMS - PFB]$^-$ carboxylate anions versus collision gas pressure at a collision energy of 30 eV:
(a) Fragmentation
(b) Collection
(c) Overall CAD

The efficiency curves for the four PGs demonstrate that differences do exist between F-series PGs and PGE_2 and PGD_2. However, by examining Figures 4-5a, 4-5b and 4-5c, similarities can be noted for certain PGs. PGD_2 has a fragmentation, collection and overall CAD efficiency behavior essentially the same as PGE_2. The behavior of $DHKF_{2\alpha}$ closely resembles that of $PGF_{2\alpha}$. The notable difference is that $DHKF_{2\alpha}$ has an E_F and E_{CAD} even lower than that of $PGF_{2\alpha}$. These results suggest that the presence of two TMS groups on the cyclopentyl ring (positions C-9 and C-11) of $PGF_{2\alpha}$ and $DHKF_{2\alpha}$ add to the stability of the $[M-PFB]^-$ carboxylate anion during CAD experiments.

Collision Pressure Study of the $[M-PFB]^-$ Carboxylate Anions

In order to test this hypothesis, the PFB ester derivatives (without methoxime or trimethylsilyl groups) of the PGs were examined. A conventional solids probe was used to introduce the derivatized PGs, since with only PFB derivatization the PGs were no longer amenable to GC separation. The CAD efficiency data from this study are shown in Figure 4-6. The fragmentation behavior (Figure 4-6a) of the carboxylate anions of the PFB-only derivatives is similar to that observed for the fully derivatized PGs (Figure 4-5a). The $PGF_{2\alpha}$/PFB and $DHKF_{2\alpha}$/PFB derivatives fragment even less efficiently than the MO/PFB/TMS derivatives at collision pressures > 2.0 mTorr N_2. This noted difference in the fragmentation efficiency of the PFB derivatives is reflected in the extremely low overall CAD efficiency (Figure 4-6c) of $PGF_{2\alpha}$ and $DHKF_{2\alpha}$.

The results from this study suggest that the presence or absence of TMS and MO groups of the fully derivatized $PGF_{2\alpha}$/PFB/TMS has little or no

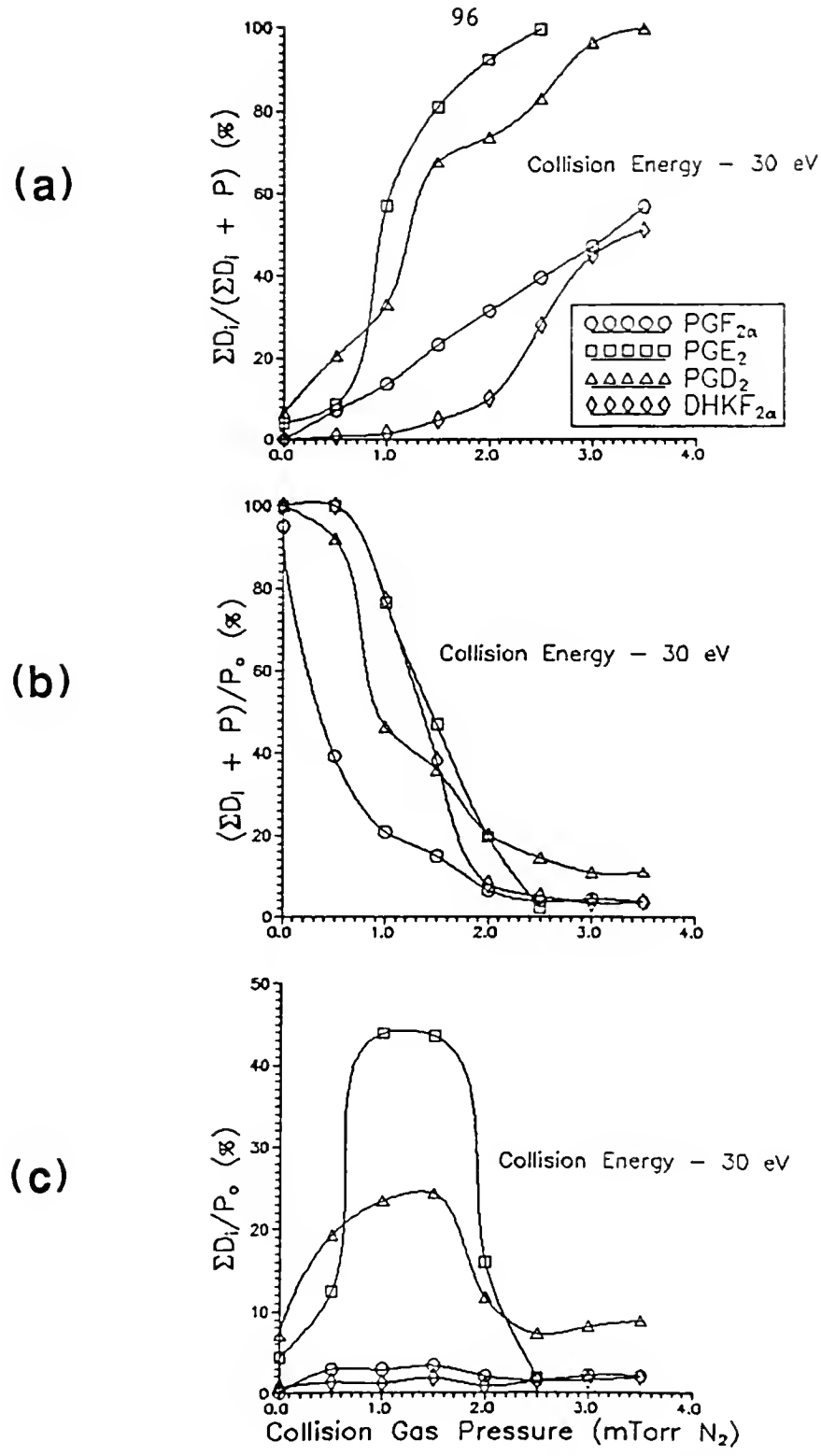
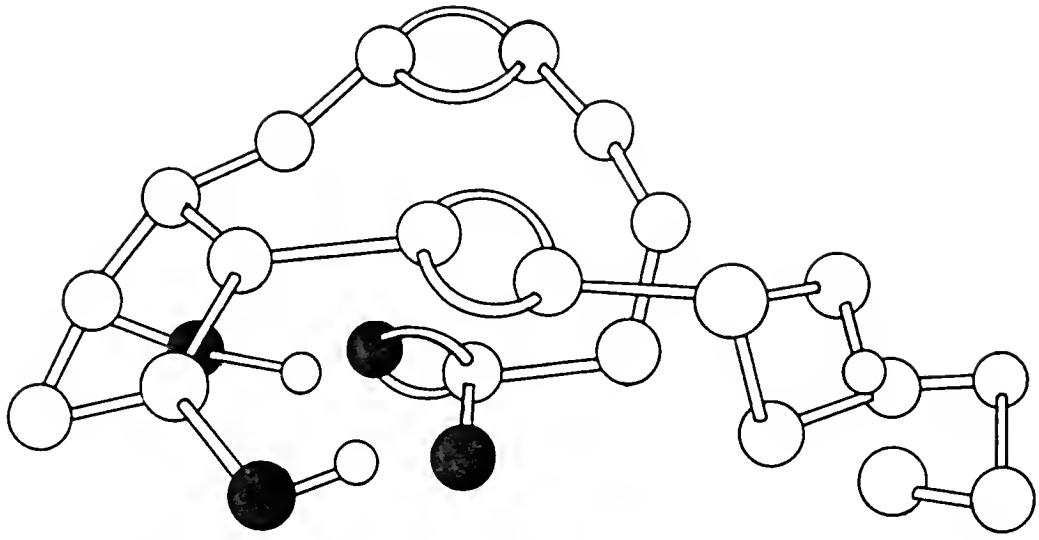
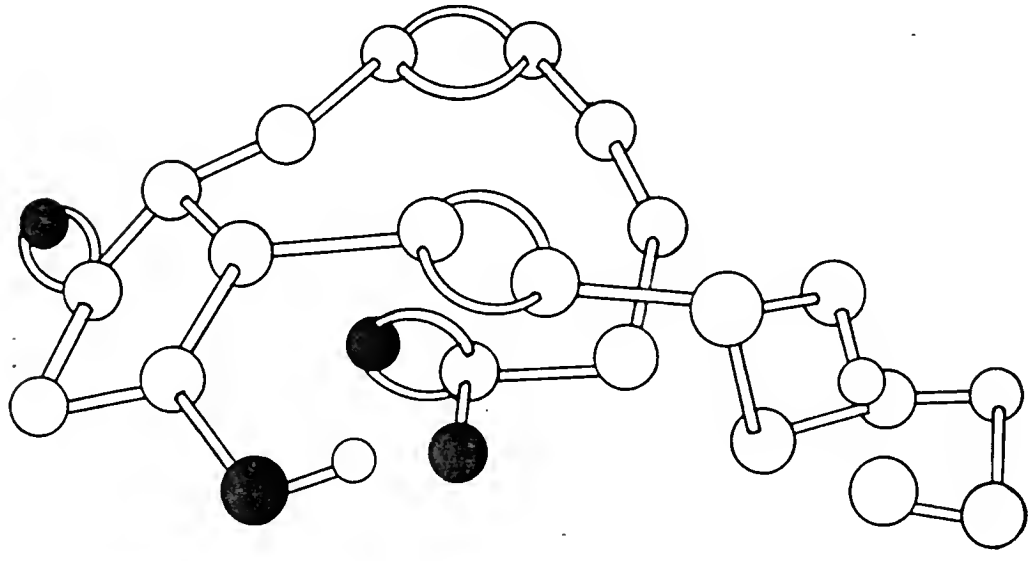


Figure 4-6: CAD efficiency of the $[M - PFB]^-$ carboxylate anions versus collision gas pressure at a collision energy of 30 eV:
(a) Fragmentation
(b) Collection
(c) Overall CAD



(a)



(b)

Figure 4-7: Sketch of ball-and-stick models of: (a) $PGF_{2\alpha}$ (b) PGE_2 Colored balls represent oxygen atoms.

effect on the observed differences in the fragmentation behavior of PGE_2 and $PGF_{2\alpha}$. It is hypothesized that the difference noted between the E_F of PGE_2 and $PGF_{2\alpha}$ is due only to the presence of two hydroxyl (or -OTMS) groups on the cyclopentyl ring of the F-series PGs. This is seen in Figure 4-7 which contains sketches of ball-and-stick models of PGE_2 and $PGF_{2\alpha}$. Figure 4-7a shows one possible configuration of $PGF_{2\alpha}$ demonstrating how the carboxylate group can interact with the two hydroxyl (OH) groups on C-9 and C-11. In contrast, the PGE_2 configuration (Figure 4-7b) shows that, with only one hydroxyl (OH) group, the interaction with the carboxylate group may not be as strong. Thus, the stability of the carboxylate anions of the F-series PGs would be expected to be greater during CAD than that of PGE_2 and PGD_2.

Collision Pressure Study of the $[M-H]^-$ Carboxylate Anions

Underivatized PGs were examined to look at the fragmentation and CAD efficiency of the $[M-H]^-$ carboxylate anions. These ions have nominally the same structures as the $[M-PFB]^-$ carboxylate anions formed by dissociative electron capture from the PFB-derivatized compounds. A conventional solids-probe was used for this study, as in the experiments done on the PFB-only derivatives ($[M-PFB]^-$). The $[M-H]^-$ ions of PGE_2 and PGD_2 could not be studied due to their low abundance (0.1% relative to $[M-H_2O]^-$) in the EC-NCI mass spectrum; however, both $PGF_{2\alpha}$ and $DHKF_{2\alpha}$ produced an $[M-H]^-$ ion intense enough to permit CAD analysis. The E_F, E_C and E_{CAD} for these underivatized $[M-H]^-$ ions are shown in Figure 4-8. The curve for E_C (Figure 4-8b) of the $[M-H]^-$ ion for $DHKF_{2\alpha}$ is similar to that found for the $[M-PFB]^-$ ion in Figure 4-6b. But, for $PGF_{2\alpha}$, at lower collision

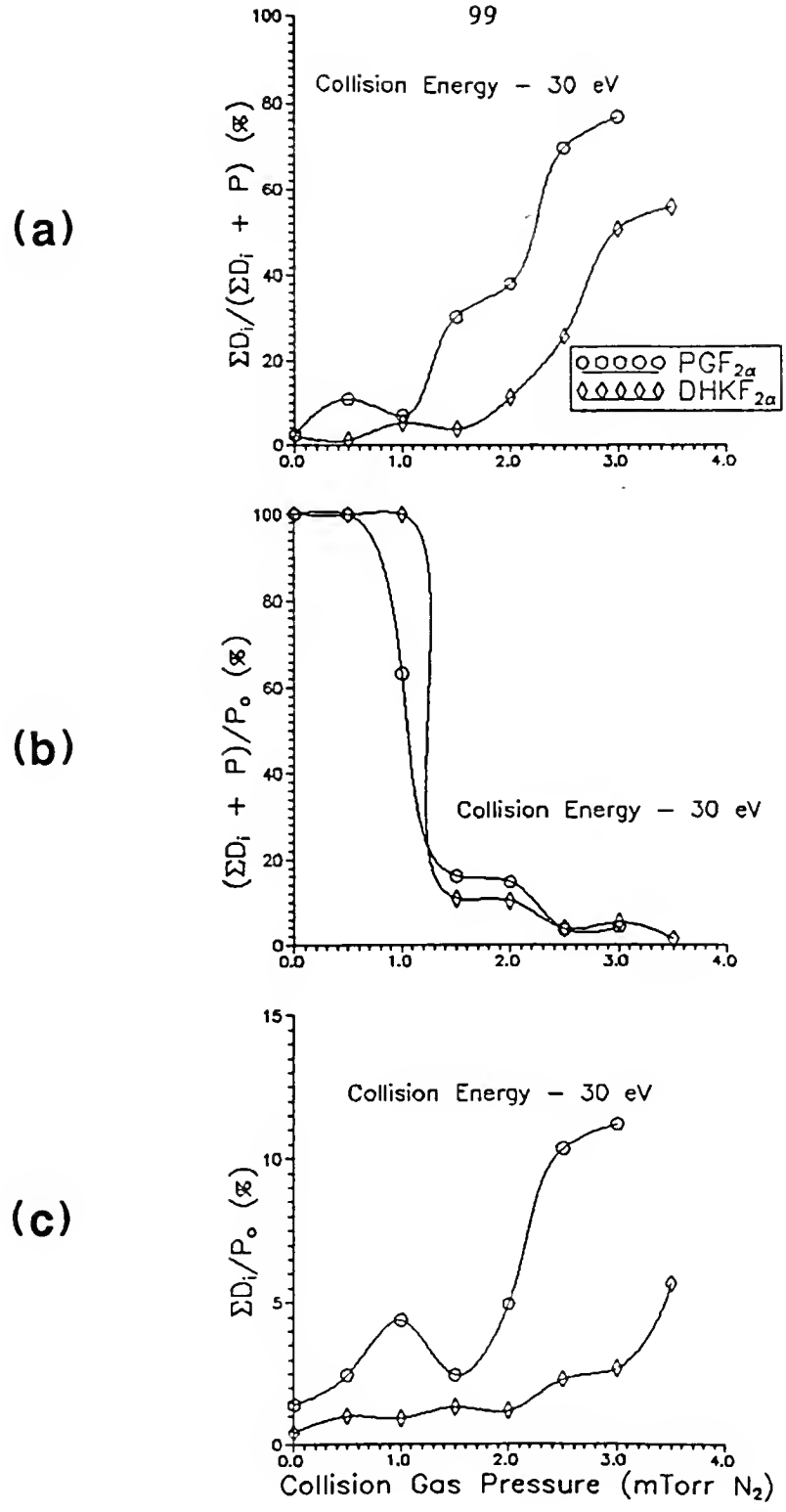


Figure 4-8: CAD efficiency of the $[M - H]^-$ carboxylate anions versus collision gas pressure at a collision energy of 30 eV:
(a) Fragmentation
(b) Collection
(c) Overall CAD

pressures (< 1.0 mTorr N_2), the E_C is 3 times higher than in the case of Figure 4-6b. Looking at Figure 4-8a and 4-8c, the fragmentation and CAD efficiencies are shown to be similar to those found in Figures 4-6a and 4-6c. The E_F and E_{CAD} for $PGF_{2\alpha}$ and $DHKF_{2\alpha}$ are 2-4 times greater in the case of the $[M-H]^-$ ion than for the $[M-PFB]^-$ ion. This slight difference between the ions may be explained by the increased internal energy in the $[M-H]^-$ ion produced under EC-NCI compared to that of the $[M-PFB]^-$ ion produced by dissociation electron capture.

Conclusions

The differences in fragmentation behavior of PGE_2 and $PGF_{2\alpha}$ were examined through the use of fragmentation, collection and overall CAD efficiency studies. We have found that the CAD efficiency for the $[MO/TMS-PFB]^-$, $[M-PFB]^-$ and $[M-H]^-$ carboxylate anions is significantly different for closely related PGs. Through the use of these curves, we have shown that F-series PGs fragment less efficiently than PGE_2 and PGD_2. A possible explanation has been proposed to explain these drastic differences seen in the fragmentation behavior of structurally similar carboxylate anions of PGs. It is believed that F-series PGs are more stable during CAD due to the interaction of the carboxylate group with the two hydroxyl (OH) groups in the cyclopentyl ring.

CHAPTER 5

EVALUATION OF SOLID-PHASE EXTRACTION GC/MS AND GC/MS/MS FOR THE ANALYSIS OF ENDOGENOUS PROSTAGLANDIN E_2 IN URINE

Introduction

The trace determination of prostaglandins (PGs) involves three basic steps; sample preparation, sample introduction and detection. A logical combination of these three steps can produce an analytical method with the required selectivity, sensitivity and total analysis time for the detection of endogenous levels of PGE_2 in urine. As was pointed out in earlier chapters, various sample preparation methods coupled with GC/MS have been reported extensively in the literature (10,11). However, the formation of the MO/PFB/TMS derivative followed by subsequent analysis with GC/electron-capture negative chemical ionization (EC-NCI) MS utilizing selected-ion monitoring (SIM) sometimes produces many interfering peaks which prevent reliable quantitation (6,17,48). These interferences, often referred to as chemical noise (91,111), can stand in the way of achieving required detection limits, or require more extensive sample clean-up.

Two methods can be used to reduce this chemical noise. Knapp and Vrbanac have taken the approach of immunoaffinity (IA) column purification with GC/EC-NCI/MS to give a very selective analysis (19,20). This method utilizes a selective sample clean-up and purification in a relatively

rapid two-step procedure to reduce the chemical noise. First, an octadecyl silyl (C18) solid-phase extraction column is employed for clean-up to conveniently concentrate a large volume of urine to a manageable volume and to provide a clear solution free of precipitates for application to the IA column (the second step in the clean-up). Further reduction of the chemical noise can be accomplished by GC in conjunction with tandem mass spectrometry (MS/MS) utilizing the selected-reaction monitoring (SRM) mode (91,111). Recently, GC/MS/MS analysis of selected PGs in biological matrices has been reported using various ionization and derivatization methods (74,76,77). The combination of both these chemical noise reduction methods has been exploited by Knapp and Vrbanac (78) in the analysis of PGs by GC/electron-impact (EI)/MS/MS using SRM. However, EI lacks the sensitivity needed in many cases for the low level determination of PG's. Investigation of all three basic steps (sample preparation, introduction and analysis) which comprise the analysis is necessary to determine the most sensitive and selective method for the determination of PGs in a biological matrix.

It is often observed that trade-offs in selectivity, sensitivity and total analysis time exist in the three basic steps of any trace analytical method. Previous work in our research group has shown the advantages and disadvantages of short 3 m and conventional 30 m GC capillary columns for GC/MS and GC/MS/MS (96). However, no systematic study has been performed for the entire analytical procedure. In this study, different combinations of sample preparation, GC column lengths and mass spectrometry detection schemes have been investigated. The trade-offs between selectivity, sensitivity and time of analysis throughout the

analytical methodology have been evaluated in this chapter. In addition, quantitation of endogenous PGE_2 in urine has been performed with several different methods.

Experimental

Prostaglandins and Reagents

All solvents were reagent or HPLC grade. Prostaglandin E_2 (PGE_2) was purchased from Sigma Chemical Co. (St. Louis, MO). The solid-phase extraction columns were purchased from Analytichem International, Inc. (Harbor City, CA). 3,3',4,4'-($^2H_4$) PGE_2 and the antibody affinity sorbent were gifts from Drs. J.J. Vrbanac and D.R. Knapp of the Department of Pharmacology, Medical University of South Carolina (Charleston, SC). The derivatization reagents and solvents pyridine, O-methylhydroxylamine hydrochloride, acetonitrile, and N,N-diisopropylethyl amine for GC/MS percent recovery studies were all purchased from Sigma Chemical Co.. Pentafluorobenzyl-bromide (PFBBr) and bis(trimethylsilyl)trifluoro-acetamide (BSTFA) were purchased from Pierce Chemical Co. (Rockford, IL). The urine was collected on two separate days from the author. All glassware was silanized with a solution of 5% dimethyldichlorosilane in toluene. These two chemicals were both purchased from Sigma Chemical Co.. Helium used as GC carrier gas, methane (>99%) used as the chemical ionization reagent gas, and argon used as CAD collision gas were from Matheson Gas Products, Inc. (Orlando, FL).

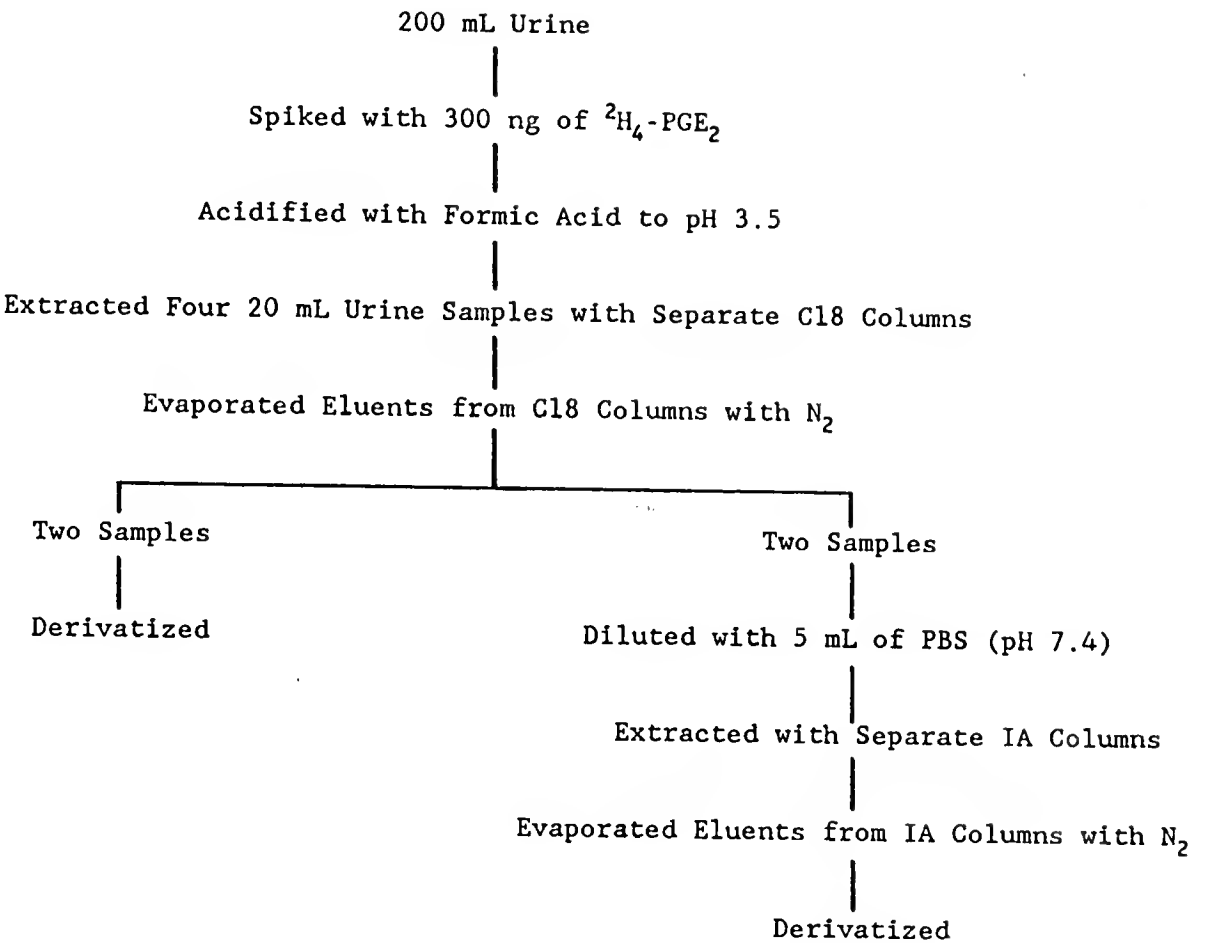


Figure 5-1: Sample preparation scheme for urine.

Sample Preparation

Two different urine specimens from different days were prepared for the quantitation study. Samples 1 and 2 were from the first urine specimen and sample 3 was from the second urine specimen. The first two urine samples were prepared according to the scheme in Figure 5-1. For sample 3 from the second urine specimen, only two C18 columns were utilized, and a different immunoaffinity (IA) column than that for samples 1 and 2 was used.

A 200 mL urine sample was acidified with formic acid to pH 3.5 after addition of 300 μL of a 1 ng/μL solution of $2H_4$-PGE_2. Extraction then followed with four 20 mL samples of the spiked urine introduced onto four separate C18 columns. Preparation and extraction of the PGE_2 utilizing the C18 columns was accomplished using the following method:

(1) Conditioned the column with 10 mL of HPLC water and 10 mL of methanol.

(2) Passed solution of PGE_2 (acidified to pH 3.5 with formic acid) through the column.

(3) Washed the column with 10 mL of HPLC water and 10 mL petroleum ether.

(4) Eluted PGE_2 with 10 mL of ethyl acetate.

After the C18 extraction all four samples were evaporated with N_2. Two of the samples were then further purified with separate IA columns. These residues were diluted with approximately 5 mL of a phosphate buffer solution (pH 7.4). This solution was then applied to the IA column and extracted as follows:

(1) Conditioned the column with 20 mL of PBS (pH 7.4).

(2) Passed solution of PGE_2 (acidified to pH 3.5 with formic acid) through the column.

(3) Allowed the sample to settle into the sorbent bed for 15 min at room temperature.

(4) Washed the column with 25 mL of PBS (pH 7.4) and 10 mL HPLC water. Removed all remaining water in the column.

(5) Eluted PGE_2 with 15 mL of 95% acetonitrile solution (v:v).

(6) Washed column with an additional 10 mL of 95% acetonitrile to assure removal of all the PGE_2.

(7) Immediately rinsed the column with 10 mL of HPLC water and 15 mL of PBS (pH 7.4).

The eluents were then evaporated with N_2.

Derivatization

The evaporated samples were then derivatized according to the procedure in Chapter 2. The amount of reagents used to derivatize the urine samples was tripled for the methoximation and PFB esterification steps compared to the amount used to derivatize standards to assure efficient derivatization of the analyte in the presence of urine matrix components which could also be derivatized.. The trimethylsilyl derivative then was formed by adding 60 μL of BSTFA to the esterfied urine samples and 50 μL to the standards for the calibration curves and allowing the solutions to stand overnight at room temperature. One-microliter injections containing 500 pg of $2H_4$-PGE_2 were made of each standard and sample.

Instrumental Conditions

A Finnigan MAT triple stage quadrupole (TSQ45) gas chromatograph/ tandem mass spectrometer was used for these studies. Gas chromatography was carried out on various lengths of J&W Scientific (Folsom, CA) DB-1 (30, 10, and 3 meter long, 0.25 mm i.d., 0.25 μm film thickness) capillary columns. The different lengths of GC columns were used in the splitless mode with helium carrier gas at the following conditions: 30 m GC column was operated at a flow rate of 41 cm/s (inlet pressure 18-20 psi) with an initial temperature of 100°C held for 30 s, increased at 25°C/min to 250°C, then increased again at 5°C/min to 310°C; the 10 m GC column was operated at a flow rate of 50 cm/s (inlet pressure 10-12 psi) with the same temperature program except for an initial temperature of 200°C; the 3 m GC column was operated at an inlet pressure of 4-6 psi, with an initial temperature of 200°C held for 30 s, then increased at 20°C/min to 260°C. The injector temperature was 300°C for all three GC column lengths.

Mass spectrometry conditions were: interface and transfer line temperature 300°C, ionizer temperature 190°C, electron energy 100 eV and emission current 0.3 mA. Electron-capture negative chemical ionization (EC-NCI) was carried out with methane at an ionizer pressure of 0.50 Torr. Argon was employed as the collision gas at a pressure of 1.0 mTorr and a collision energy of 25 eV was utilized for the selected-reaction monitoring studies of PGE_2. The GC/MS analysis utilizing SIM used an electron multiplier (EM) setting of 1500 V and a preamp gain of 10^8 V/A. GC/MS/MS experiments with SRM employed an EM setting of 2000 V and a preamp gain identical to the SIM analysis.

Table 5-1: Analytical Schemes Used for the Determination of Endogenous Levels of PGE_2 in Urine

Method	Sample Preparation	GC Column Length	Detection Method
A[a]	C18 Column	30 meter	SIM[b]
B[a]	C18 Column	30 meter	SRM[c]
C[a]	C18 & IA[d] Columns	30 meter	SIM
D[a]	C18 & IA Columns	30 meter	SRM
E	C18 Column	10 meter	SIM
F	C18 Column	10 meter	SRM
G[a]	C18 & IA Columns	10 meter	SIM
H[a]	C18 & IA Columns	10 meter	SRM
I	C18 Column	3 meter	SRM
J	C18 & IA Columns	3 meter	SIM
K[a]	C18 & IA Columns	3 meter	SRM

[a] Methods employed for quantitation study of the endogenous level of PGE_2 in urine.
[b] Selected-ion monitoring
[c] Selected-reaction monitoring
[d] Immunoaffininty purification

Eleven different analytical schemes were investigated for the study of PGE_2 in urine. These methods are listed in Table 5-1 with the sample preparation, GC column length and the mass spectrometric analysis method employed. Seven of the methods examined were employed for the quantitation of the endogenous levels of PGE_2 in urine. The schemes selected for quantitation are noted in the table.

Standard calibration curves for the seven quantitation methods were prepared by analyzing solutions containing a constant amount of $^2H_4$-PGE_2 (25 ng) and increasing amounts of PGE_2. Table 5-2 lists the amounts of $^2H_4$-PGE_2 and standard PGE_2 added to each vial and the final concentrations after dilution with 50 μL of silylating reagent. An example of a calibration curve that was obtained with a 10 m GC column utilizing SIM, is shown in Figure 5-2. Six different calibration curves were obtained for the three lengths of GC columns employed utilizing either SIM or SRM. The calibration curve shown demonstrates good linearity with a slope of 1.06, similar to the expected value of 1.00 for this calibration curve. The five other calibration curves obtained exhibit similar linearity with slopes similar to that found for the calibration curve shown in Figure 5-2. Extrapolation of the x-intercept from the best fit line yields the amount of PGE_2 in 500 pg of the $^2H_4$-PGE_2 internal standard. The average intercept obtained from the six calibration curves corresponded to approximately 6 pg of PGE_2 which is 1.1% of the 500 pg of $^2H_4$-PGE_2.

The quantitation of the endogenous levels of PGE_2 in the extracted urine sample was accomplished by selected-reaction monitoring (SRM) and selected-ion monitoring (SIM). Two gas chromatographic peaks appear for derivatized PGE_2 in the chromatograms in this chapter. These correspond

Table 5-2: Calibration Curve Dilutions for the Quantitation of PGE_2

$^2H_4$-PGE_2 Added (ng)	PGE_2 Added (ng)	Volume of Dilution (μL)	Concentration of $^2H_4$-PGE_2 (pg/μL)	Concentration of PGE_2 (pg/μL)
25	0.0	50	500.0	0.0
25	1.0	50	500.0	20.0
25	2.5	50	500.0	50.0
25	5.0	50	500.0	100.0
25	10.0	50	500.0	200.0
25	20.0	50	500.0	400.0
25	25.0	50	500.0	500.0

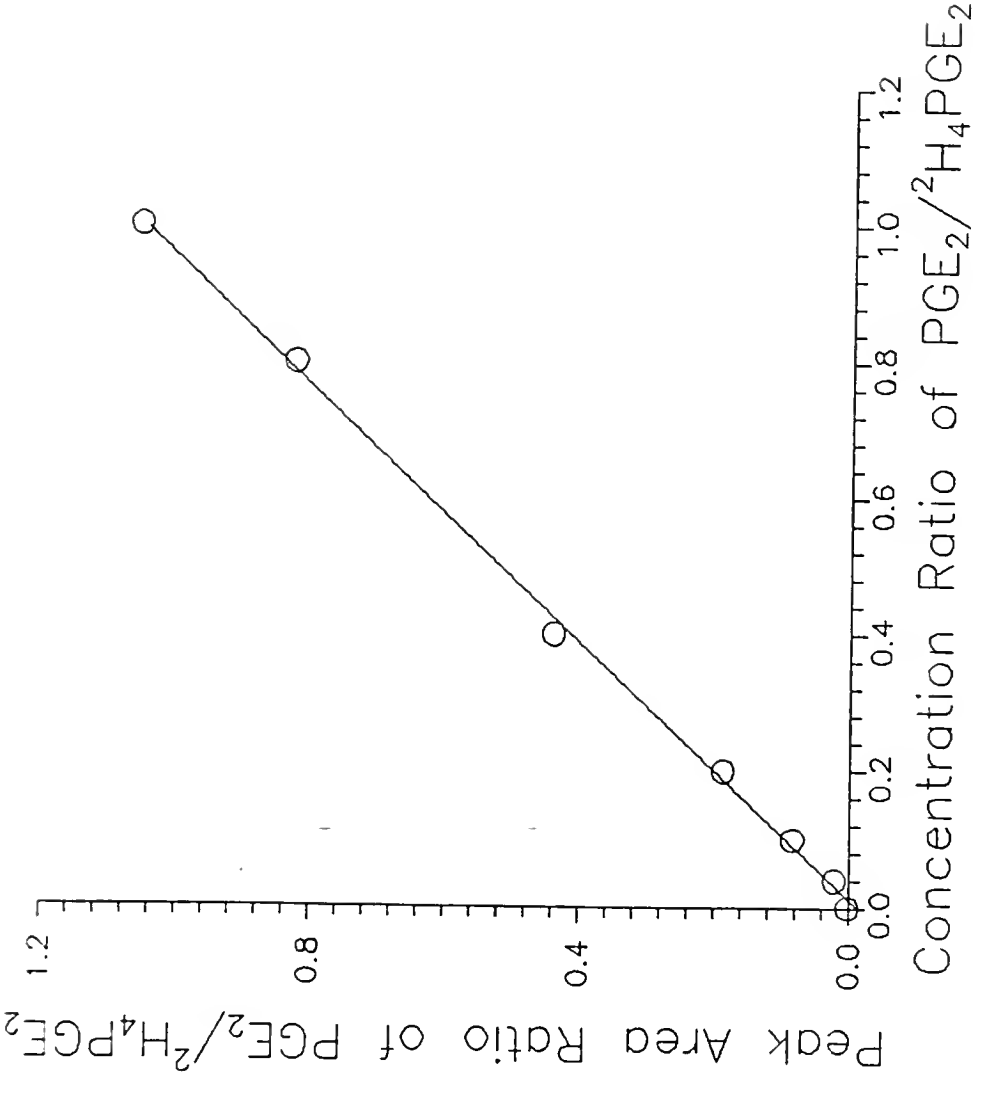


Figure 5-2: Calibration curve utilizing a 10 m GC column with SIM.

to the syn- and anti-methoxime isomers of the derivatized PGE_2 (75). The major anti-methoxime isomer of PGE_2 (the second peak) was used for quantitation in these studies. The $[M-PFB]^-$ ions of PGE_2 (524^-) and $^2H_4$-PGE_2 (528^-), were monitored for selected-ion monitoring studies. Optimum conditions from Chapter 3 were used for the analysis of PGE_2 and $^2H_4$-PGE_2. The selected-reactions monitored for the analysis of endogenous levels of PGE_2 in urine were $524^- \rightarrow 268^-$ for PGE_2 and $528^- \rightarrow 272^-$ for the internal standard $^2H_4$-PGE_2. A baseline was chosen visually on the GC trace and the peak areas for PGE_2 and $^2H_4$-PGE_2 calculated by the INCOS computer system for the standards and the extracted urine samples. The area of PGE_2 divided by the area of $^2H_4$-PGE_2 in the standards gives a ratio that is used in the calibration curve. The amount of PGE_2 in each urine sample was calculated by comparing the ratio of these ions to that of the calibration curve. A typical SIM chromatogram (30 m GC) of a urine extract (cleaned-up by C18 and IA) is shown in Figure 5-3a; the results with only C18 clean-up are shown in Figure 5-3b. The amount of PGE_2 in the sample is calculated from the ratio of the peak area (shaded in black) in the top trace to that of $^2H_4$-PGE_2 in the bottom trace. The $^2H_4$-PGE_2 is used both as an internal standard for quantitation and as a retention time marker for identification of the endogenous PGE_2 peak which may not be fully resolved, as in Figure 5-3b. Note that the derivatized $^2H_4$-PGE_2 elutes approximately 5 to 6 seconds before derivatized PGE_2. The amount of PGE_2 found from the calibration curve was then multiplied by the dilution factor of 60. This indicates the amount of endogenous PGE_2 in the 20 mL urine sample. Dividing this amount by 20 results in the amount of endogenous PGE_2 per mL of urine.

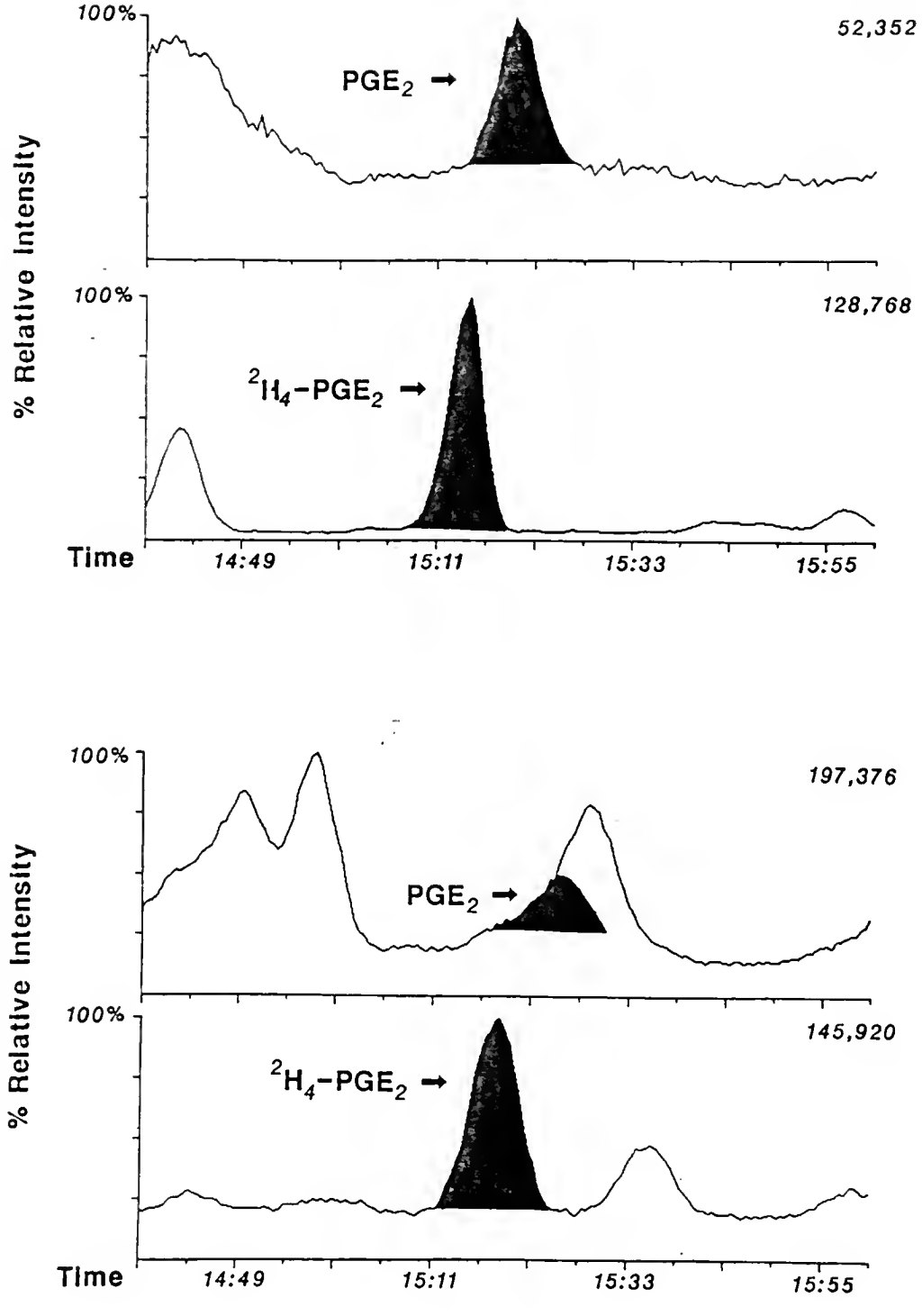


Figure 5-3: Typical SIM chromatogram of a urine extract showing both the endogenous PGE_2 (524^-) and $^2H_4$-PGE_2 (528^-) with:
(a) C18 and IA purification (b) C18 extraction only
The integrated peak areas are shaded in black.

Results from the Quantitation Study of PGE_2 in Urine

Quantitation Study

The seven analytical schemes employed for quantitation of PGE_2 in urine and the results of the study are shown in Table 5-3. The three samples discussed earlier were analyzed by each method on the same day. Three injections of each sample were made and the average and %RSD calculated. In addition, the average of samples 1 and 2 (from the same urine specimen) is listed, as well as the average of all three samples and the %RSD.

This table provides a great deal of information about the methods used for the quantitation of PGE_2 in urine. First, the quantities found for the endogenous PGE_2 in urine agree well with normal values reported in the literature (71,77,78). Reports have shown that the concentration of PGE_2 in urine of various subjects ranges from 100 to 400 pg/mL. Quantities detected in this study with the seven different analytical schemes all are within this range. The average value of the seven different methods was 315.2 pg with a %RSD of 3.7%. The average values of the different methods employed varied only slightly, ranging from 302.4 pg/mL to 330.8 pg/mL. This indicates that all the selected methods may be employed for the analysis of PGE_2 in urine at these levels.

The variation in the seven methods is observed in the %RSD found from averaging the results from the seven different methods employed for the three urine samples. These average values vary only slightly, with a %RSD ranging from 3.5% for urine sample 2 to a high of 5.3% for urine sample 3. In addition, this table provides information about the various

Table 5-3: Quantitation Study of Endogenous PGE_2 in Urine Utilizing Various Analytical Schemes

Method[a]	Sample #	Quantity of PGE_2 (pg/mL) GC Injection #			Average of 3 Inj.	%RSD[b]	Average of 1 & 2	Average of All 3	%RSD of all 3
		1	2	3					
	1	363.2	348.3	360.3	357.3	2.2	337.0		
A	2	321.8	310.5	317.6	316.6	1.8		327.8	7.9
	3	312.8	307.2	308.8	309.6	0.9			
	1	363.0	372.8	352.1	362.6	2.9	340.7		
B	2	326.0	320.5	309.6	318.7	2.6		319.9	13.1
	3	279.6	270.1	285.2	278.3	2.7			
	1	344.8	332.7	317.5	331.7	4.1	322.3		
C	2	302.5	321.8	314.9	312.9	3.1		302.4	11.8
	3	262.0	267.7	259.6	262.7	1.5			
	1	348.5	371.5	313.7	344.6	8.4	329.2		
D	2	323.0	304.3	313.7	313.7	3.0		316.3	8.6
	3	284.1	292.3	295.7	290.7	2.1			

[a] Refer to Table 5-1 for explanation of analytical schemes.
[b] % Relative Standard Deviation

Table 5-3: --continued

Method[a]	Sample #	Quantity of PGE_2 (pg/mL) GC Injection # 1	2	3	Average of 3 Inj.	%RSD[b]	Average of 1 & 2	Average of All 3	%RSD of all 3
G	1	379.1	364.7	344.5	362.8	4.8	348.6		
	2	345.1	333.8	324.3	334.4	3.1		330.8	10.3
	3	299.6	297.4	288.6	295.2	2.0			
H	1	338.7	314.8	342.6	332.0	4.5	314.3		
	2	287.4	322.3	280.0	296.6	7.6		303.1	8.7
	3	270.9	266.5	304.5	280.6	7.4			
K	1	376.5	295.2	347.7	339.8	12.1	326.4		
	2	273.7	267.3	364.2	312.9	18.0		306.0	10.4
	3	285.3	264.4	279.5	276.4	3.9			

Urine Sample #	Average from 7 Methods (pg/mL)	%RSD
1	347.3	3.9
2	315.1	3.5
3	284.8	5.3

[a] Refer to Table 5-1 for explanation of analytical schemes.
[b] % Relative Standard Deviation

sample preparations and mass spectrometric analysis methods employed.

For example, comparing the concentrations found by method G (C18 + IA + 10 m GC + SIM) and method B (C18 + IA + 30 m + SRM) a difference is noted between the SIM and SRM analysis methods. The method utilizing SIM yielded concentrations for endogenous PGE_2 approximately 10% higher than for the method utilizing SRM. This may be due to the interferences that are present in the SIM chromatogram overlapping with the PGE_2 peak, creating difficulty in the accurate determination of the actual PGE_2 peak area. The use of SRM eliminates many of the chemical interferences, thus reducing the problem of inaccurate peak area determination.

Another important difference observed in the table occurs between methods A (C18 + 30 m GC + SIM) and C (C18 + IA + 30 m GC + SIM) for all three urine samples. The concentrations of endogenous PGE_2 determined by method A are consistently 10% higher than those found by method C. This may be explained by the removal of urine matrix components with the addition of IA in method C, permitting more accurate calculation of the PGE_2 peak area. Note that the additional selectivity of SRM (methods B and D) reduces the need for IA clean-up, therefore reducing the difference between the results obtained from methods B and D.

Limit of Detection (LOD) and Sensitivity Studies

Table 5-4 gives the approximate LOD found for $^2H_4$-PGE_2 in urine and the sensitivity values (slopes of the calibration curves in counts/pg) for PGE_2 standards. The LOD study was accomplished by diluting the urine extracts used above containing 500 pg/μL of $^2H_4$-PGE_2 to 100, 50, 25 and 13 pg/μL and monitoring $^2H_4$-PGE_2 peak area. The LODs reported in Table

Table 5-4a: Limit of Detection Study of $^2H_4$-PGE_2 in Urine for Various Analytical Schemes

Method[a]	Limit of Detection[b] (pg/mL)
A	20
B	20
C	20
D	35
G	20
H	75
K	25

Table 5-4b: Sensitivity Study of PGE_2 Standards Utilizing the Slopes of the Calibration Curves

	Slope or Sensitivity (counts/pg)	
GC Column Length	SIM	SRM
30 m	0.032[c]	0.0089[d]
10 m	0.145[c]	0.031[d]
3 m	0.805[e]	0.028[e]

[a] Refer to Table 5-1 for explanation of analytical scheme.
[b] Approximation from the dilution of the extracted urine samples to produce a peak area 3 times that of a diluted urine blank.
[c] Electron multiplier setting at 1500 V.
[d] Electron multiplier setting at 2000 V.
[e] Electron multiplier setting at 1700 V.

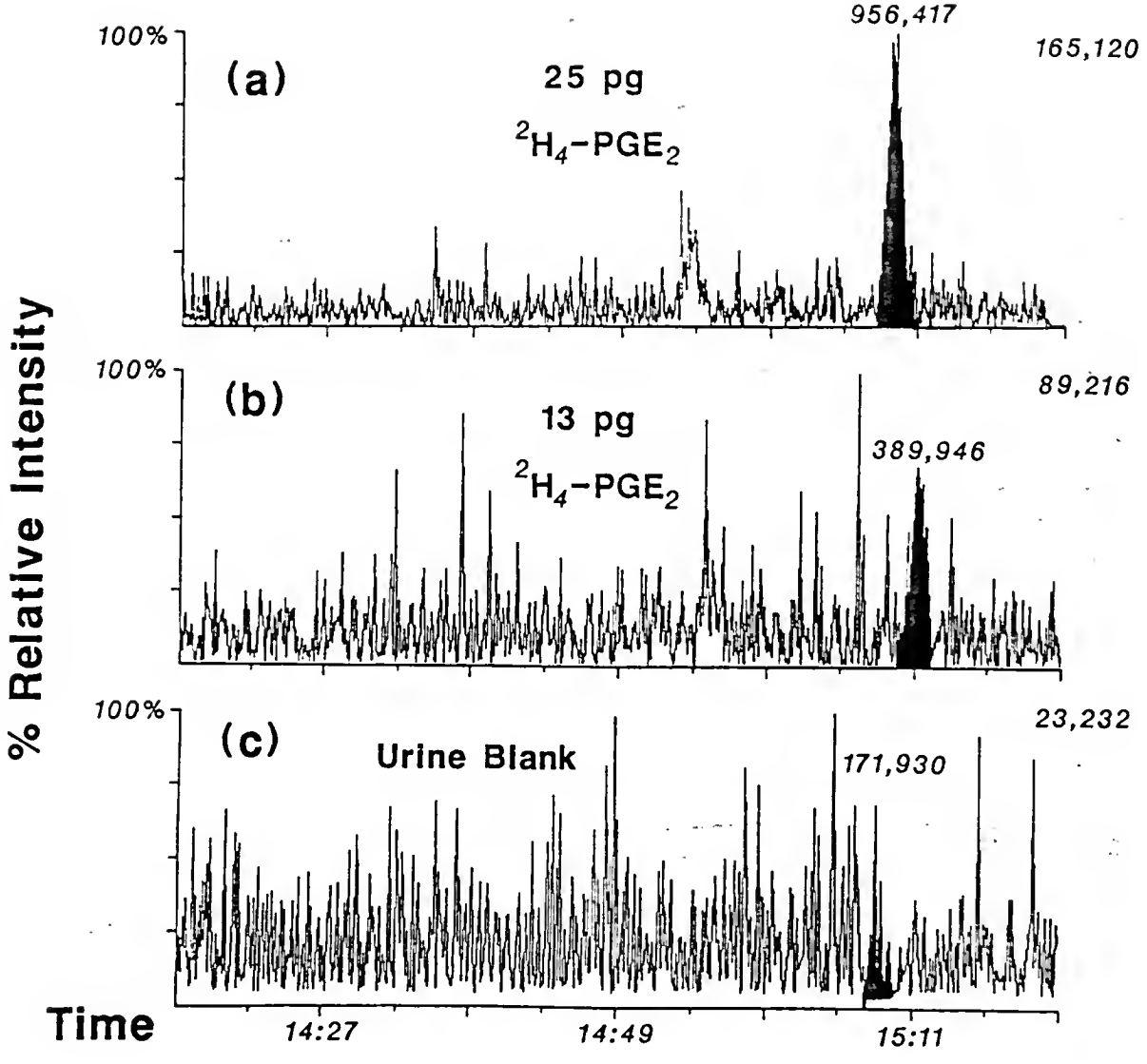


Figure 5-4: SRM chromatograms of $^2H_4$-PGE$_2$ in urine extracted with a C18 column and separated with a 30 m GC column and diluted to yield: (a) 25 pg $^2H_4$-PGE$_2$ (b) 13 pg $^2H_4$-PGE$_2$ (c) Urine blank (no $^2H_4$-PGE$_2$)

5-4a are the concentrations of $^2H_4$-PGE_2 in urine which produce a peak area three times the area calculated from a blank urine sample containing no $^2H_4$-PGE_2 diluted with the same procedure. An example of a LOD calculation (for method B) is shown in Figure 5-4, with the peak areas used for the calculation marked off on the three chromatograms. Figure 5-4a shows the SRM chromatogram of 25 pg injected on column that produces a peak area approximately 5 times the peak area of the blank. Figure 5-4b shows the SRM chromatogram of 13 pg of $2H_4$-PGE_2 which produces a peak area approximately twice that of the urine blank shown in Figure 5-4c. Examination of the LODs in Table 5-4a show interesting trends. First, comparing methods C and D or G and H, it is apparent that utilization of SRM in a case where the clean-up is already highly selective (i.e., IA) only serves to worsen the LOD. Comparing methods H and K, however, indicates that the best limits of detection can be regained with SRM by simple reducing the amount of GC separation that is employed. These results simply confirm the fact that the LOD in a real sample is a function of both sensitivity and selectivity. Whether adding another step to enhance the selectivity (e.g., SRM) with the concomitant loss in sensitivity (e.g., the less-than unity CAD efficiency and transmission efficiency through a second mass analyzer) will improve or degrade the LOD is a function of the interferences which remain in the sample. Differences in the LOD for various sample preparations, GC column lengths and mass spectrometric detection modes are discussed in detail throughout this chapter.

The slopes of the six calibration curves for PGE_2 standard utilizing SIM and SRM with 3 different column lengths are listed in Table 5-4b.

Note that not all of these sensitivities can be directly compared due to the various electron multiplier voltages employed. Comparison of the slopes for SRM and SIM using a 3 m GC column yields a sensitivity ratio of 0.035 or 3.5%. This corresponds to approximately a 30 times decrease in sensitivity for SRM analysis compared to SIM. Differences in the sensitivity for the various GC column lengths will be discussed in detail in a later section of this chapter.

Summary of Results

The concentrations of endogenous PGE_2 in urine found utilizing the seven different analytical schemes agrees favorably with those in the literature. All the results are similar, with an average of 315.2 pg/mL and a %RSD of 3.7%. The LOD varies from a low of 20 pg for four of the methods, including C18 and IA purification coupled to a 10 m GC column with SIM, to a high of 75 pg for C18 and IA purification coupled to a 10 m GC column with SRM. These data indicate that any of these methods could be used for the analysis of normal levels of urinary PGE_2.

Trade-offs in the Steps of the Analytical Procedure

The sample preparation, sample introduction and mass spectrometric detection method all affect the overall selectivity, sensitivity and the time of analysis for the determination of PGE_2 in urine. The eleven different analytical schemes listed in Table 5-1 were used to evaluate the trade-offs which result from various combinations of the three basic steps of the analytical procedure. This systematic study was then used to determine the optimum analytical scheme for the analysis of PGE_2 in urine.

Sample Preparation

The differences in sample preparation between a simple C18 extraction only and a C18 extraction with IA purification is shown in Figure 5-5. This analysis employed a 30 m GC column with SIM. These chromatograms demonstrate the additional selectivity enhancement of employing IA purification with a simple C18 extraction. In Figure 5-5a, a large number of interfering components from the urine matrix remain at concentrations much higher than PGE_2, which are not present in the case of Figure 5-5b utilizing C18 and IA. This can be observed by examining the absolute counts obtained for the chromatogram in Figure 5-5a that are approximately 13 times higher than that observed for Figure 5-5b for the major urine matrix components still present. In addition, the endogenous PGE_2 in not completely separated from the interferences present in the urine Figure 5-5a. Figure 5-5b demonstrates the effectiveness of IA purification to separate urine matrix components from endogenous PGE_2. The advantage of utilizing IA purification is even greater when employing shorter lengths of GC columns. Figure 5-6 shows the differences of these same sample preparations using a 10 m GC column with SIM. Figure 5-6a is the chromatogram employing only C18 extraction. This indicates the many interferences which are still present from the urine and are not distinguishable from the endogenous PGE_2. In addition, the concentrations of the interferences in Figure 5-6a that remain produce an absolute count value 5 times higher than that observed in Figure 5-6b. However, even with a 10 m GC column using SIM, PGE_2 can be separated from components in the urine matrix by the addition of IA purification (Figure 5-6b).

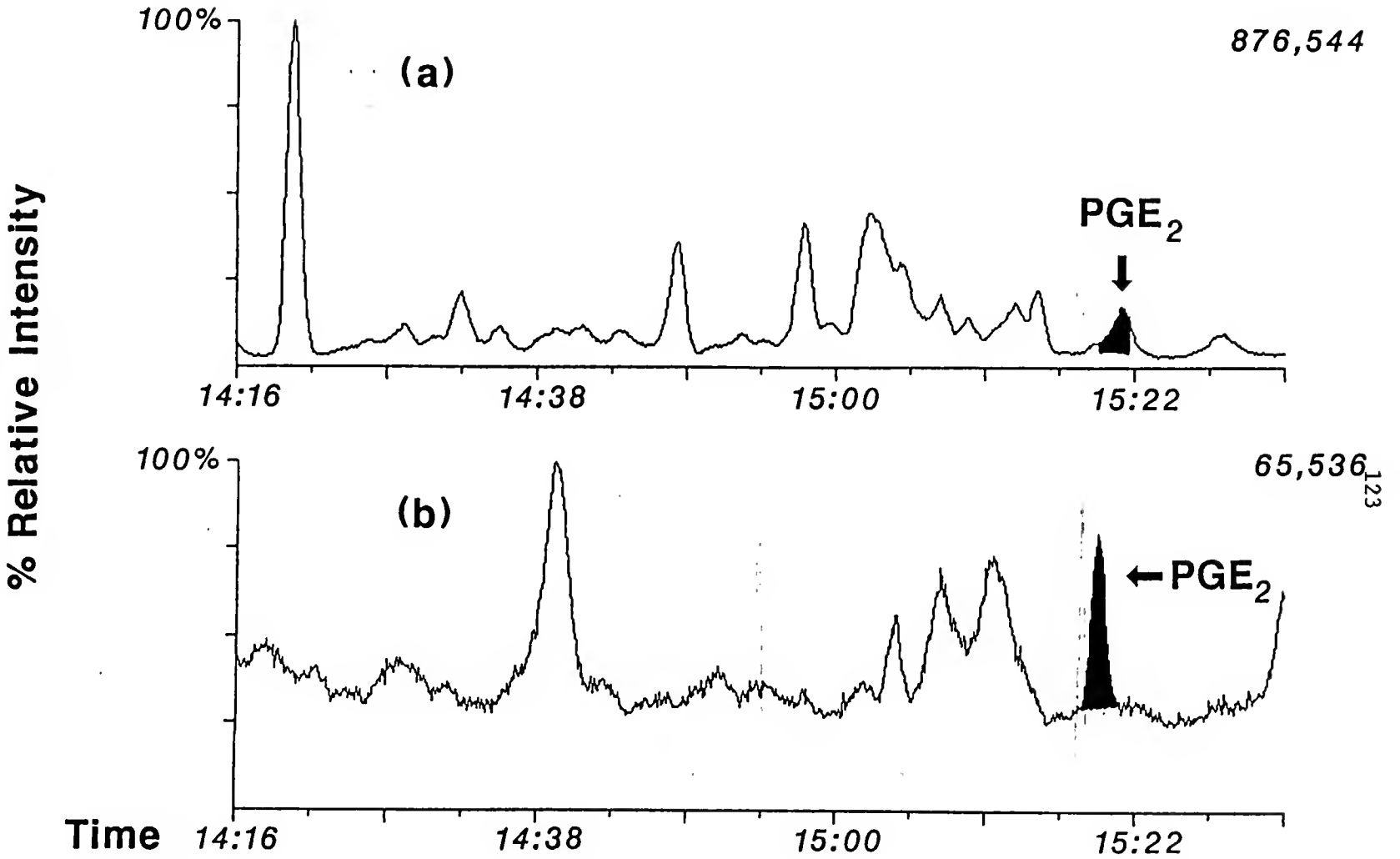


Figure 5-5: 30 m GC column SIM chromatograms of endogenous PGE_2 in urine extracted with: (a) C18 column (b) C18 & IA columns

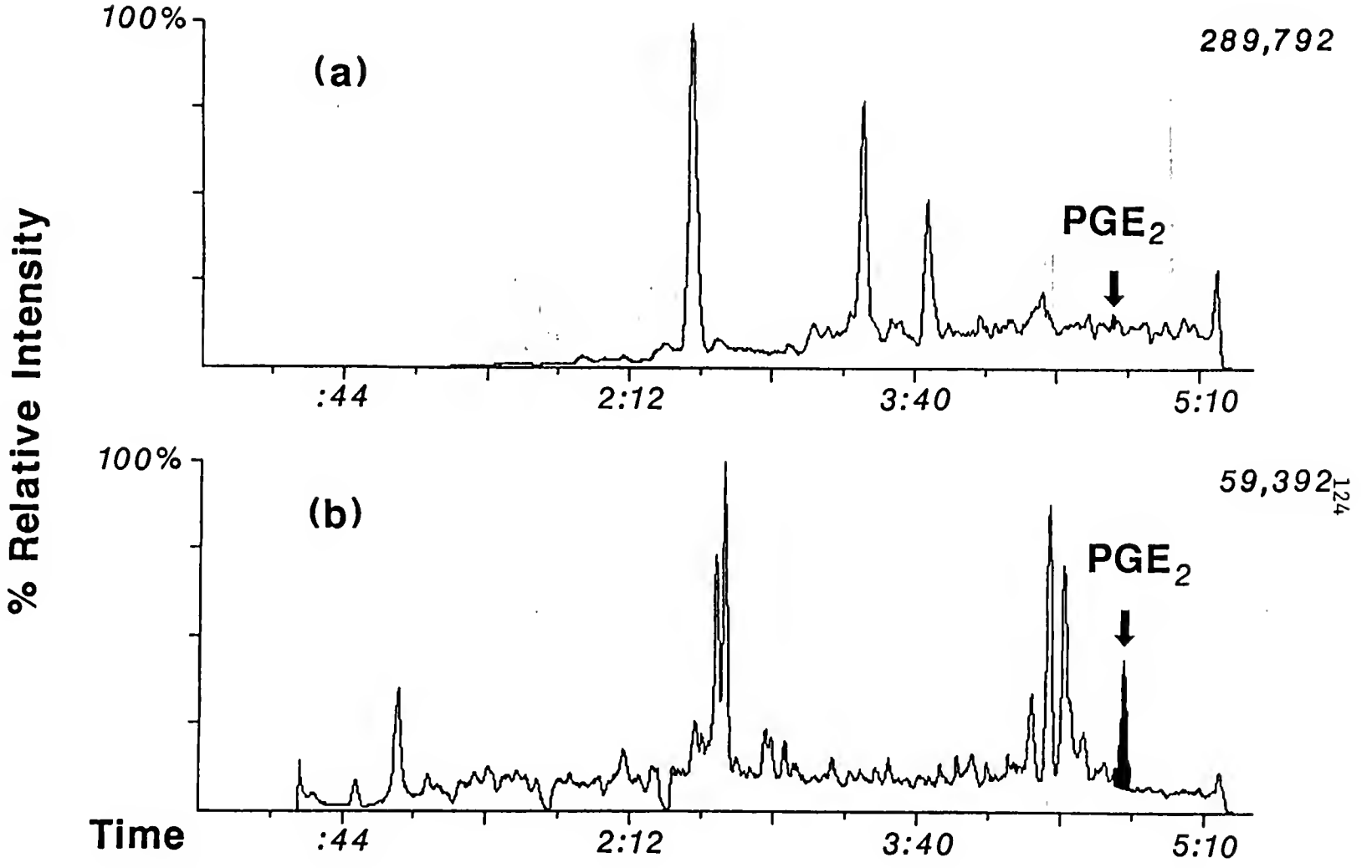


Figure 5-6: 10 m GC column SIM chromatograms of endogenous PGE_2 in urine extracted with: (a) C18 column (b) C18 & IA columns

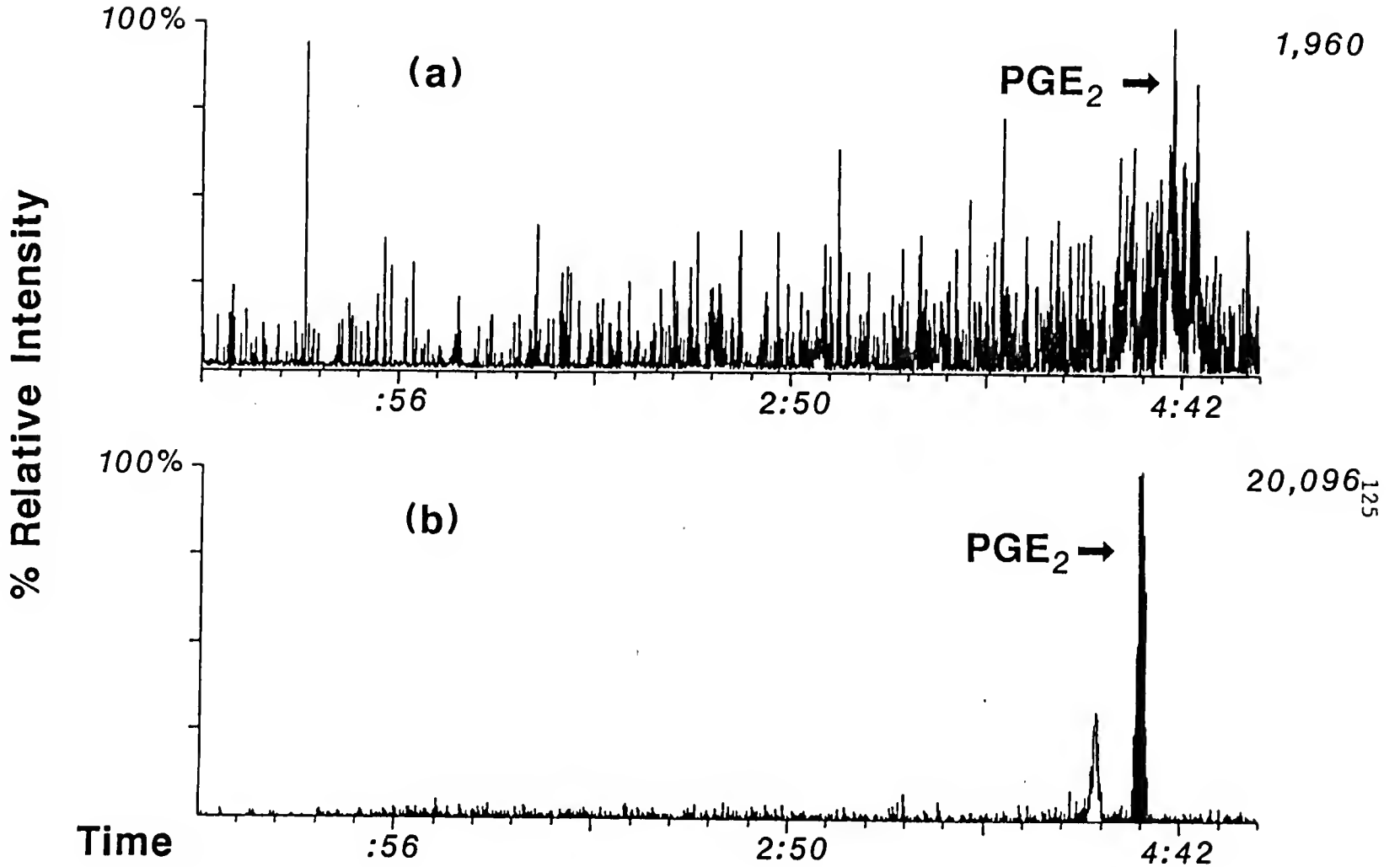


Figure 5-7: 10 m GC column SRM chromatograms of endogenous PGE_2 in urine extracted with: (a) C18 column (b) C18 & IA columns

If a different mass spectrometric technique is employed, the differences between the two sample preparations remain prominent. In Figure 5-7, separation on a 10 m GC column is used with SRM. The chromatogram utilizing only C18 extraction (Figure 5-7a) shows a reduction in the chemical noise due to the use of MS/MS, but with the lack of separation of the 10 m GC column even SRM cannot give adequate detection of endogenous levels of PGE_2 in urine. Figure 5-7b demonstrates that with C18 and IA sample preparation and the additional reduction in chemical noise by utilization of SRM PGE_2 in urine can be easily detected.

The sensitivity and selectivity of the different sample preparations are reflected in the limits of detection (LOD) in Table 5-4a. The results obtained from this study show no difference in the LOD for only the C18 extraction and the C18 and IA purification when utilizing a 30 m GC column with SIM. The LOD found for C18 and IA purification using a 30 m with SRM (35 pg) was slightly worse than for only C18 extraction with the same conditions. In theory, if the C18 extraction alone with 30 m GC/MS/MS provided adequate separation of urine matrix components from the endogenous PGE_2, then addition of another (unneeded) extraction step (IA) only serves to reduce the sensitivity due to less than 100% recovery of the PGE_2 in urine.

Another important factor to consider is the time to prepare the sample before the derivatization. The C18 extraction is simple and rapid, only requiring approximately 10 to 15 minutes for a 20 mL urine sample to be prepared. The additional IA purification requires approximately 30 minutes per sample, thus an entire sample preparation time of 40 to 45 minutes. However, the IA purification is still more rapid than

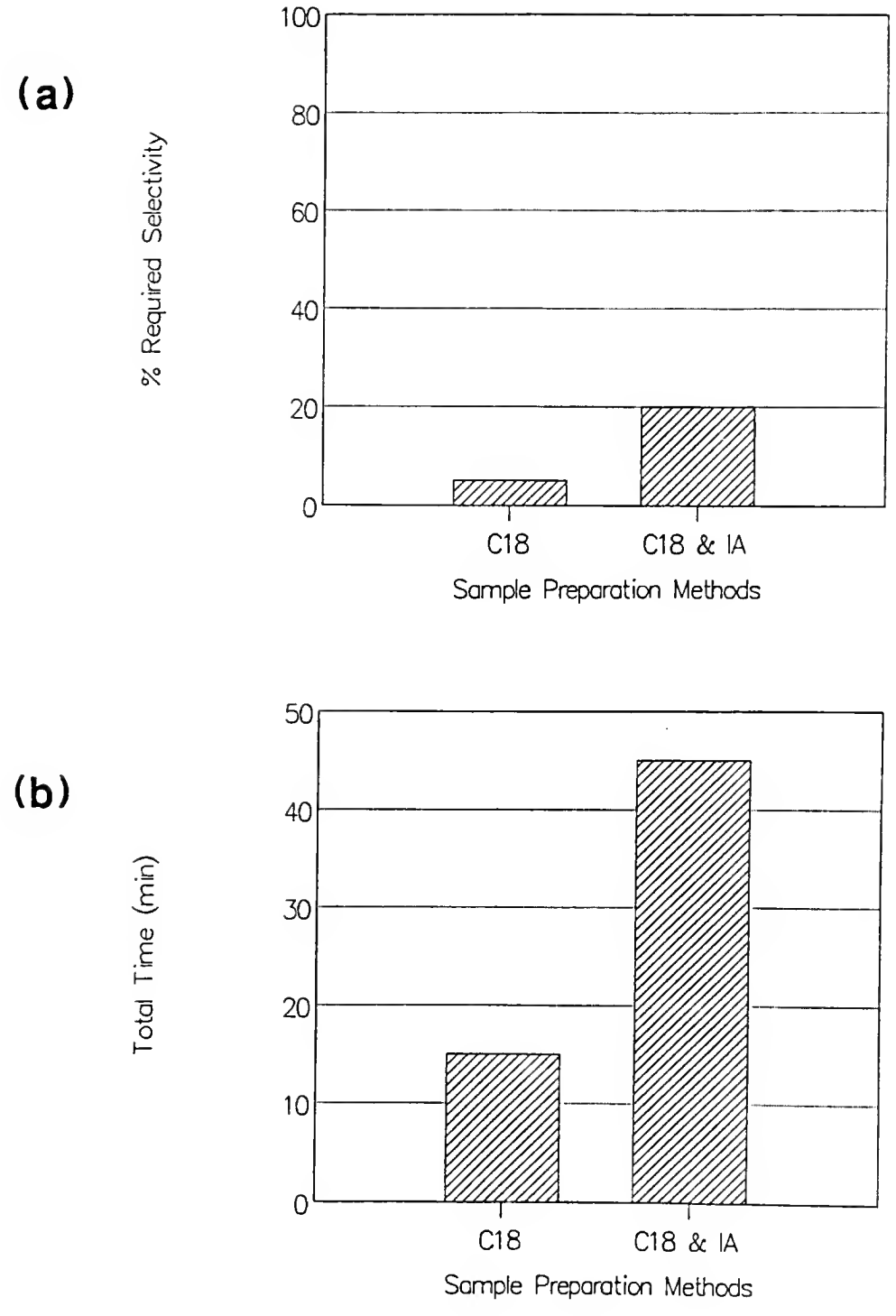


Figure 5-8: Summary of the relative differences in sample preparation methods for: (a) Selectivity (b) Total time

conventional chromatographic purification methods. Furthermore, the additional time for the IA step is a worthwhile trade-off for the considerable gain in selectivity that is achieved by this method.

The differences in relative selectivity and time of analysis are summarized in Figure 5-8. The top line on Figure 5-8a represents the selectivity required to detect endogenous levels of PGE_2 in urine (approximately 100 pg/mL). All the methods employed for the quantitation study possessed adequate limits of detection to detect endogenous levels of PGE_2 in urine. Figure 5-8b shows the time required for a C18 extraction compared to C18 and IA purification. The optimum is obviously the one which is the most rapid, possessing adequate selectivity for reliable analysis.

Sample Introduction

The various lengths of GC columns employed demonstrate considerable differences in selectivity. Figure 5-9 shows the use of C18 and IA sample preparation coupled to 30 m, 10 m and 3 m GC columns with SIM. Differences in the separation of urine matrix components from endogenous PGE_2 is evident in the chromatograms. The additional separation ability of a 30 m GC column (Figure 5-9a) increases the selectivity of the method, albeit at a cost of longer analysis time. Ultimately, a 30 m GC column possess the greatest selectivity for this analysis. However, as was discussed previously, the utilization of C18 and IA for sample preparation produces a clean enough sample that selectivity (separation) of a 10 m GC column is adequate for separation of urine matrix components from PGE_2. Figure 5-9c shows that with this method of analysis, however,

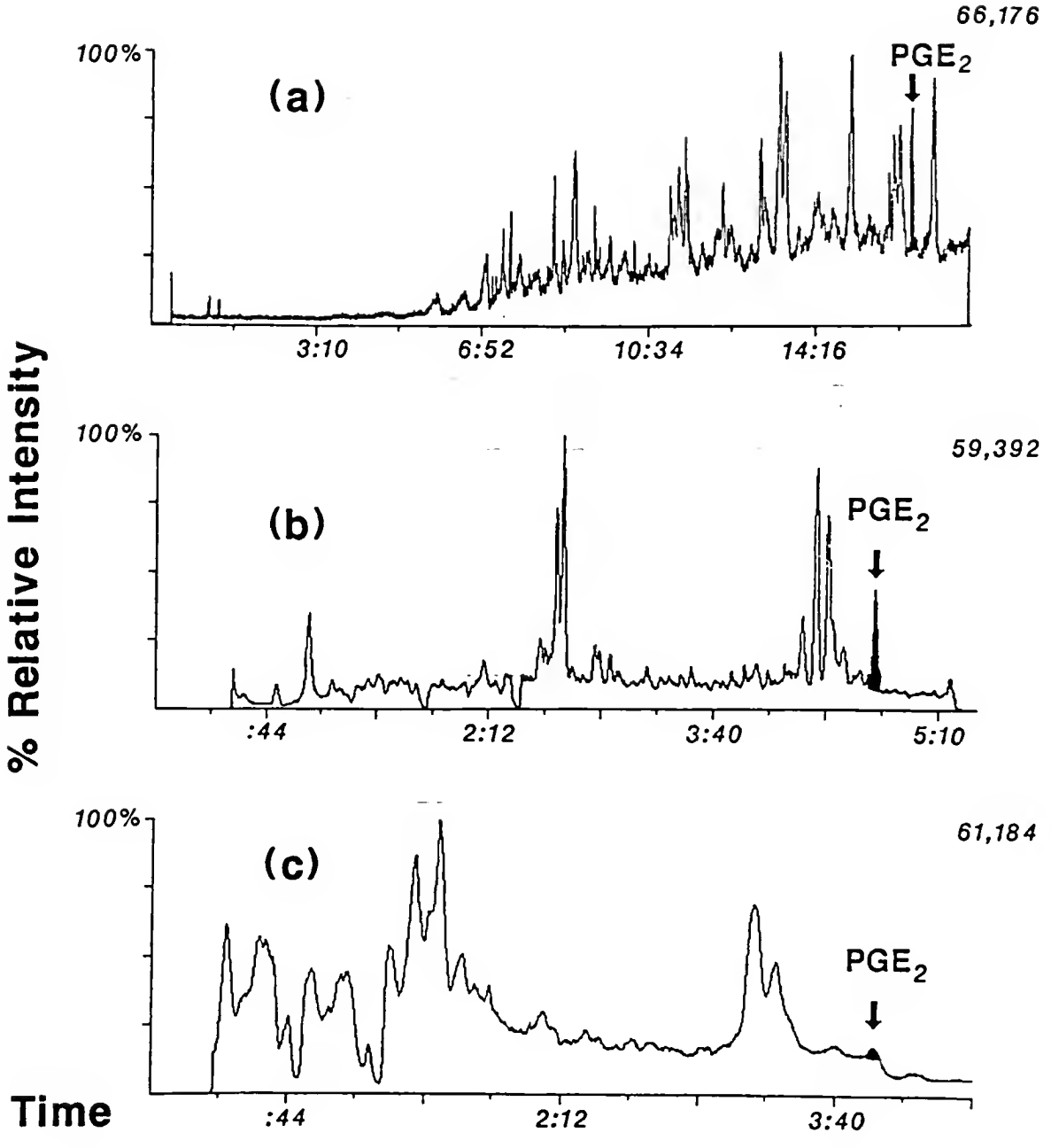


Figure 5-9: SIM chromatograms of endogenous PGE_2 in urine extracted with C18 and IA columns and separated with:
(a) 30 m GC (b) 10 m GC (c) 3 m GC

a 3 m GC column does not possess adequate selectivity for the analysis of PGE_2 in urine.

The sensitivity and selectivity for the different lengths of columns with different analysis methods are reflected in the detection limits presented in Table 5-4a. In theory, if the same temperature programs were employed for the three lengths of GC columns, a 3 m column should produce shorter retention times and possibly narrower, taller peaks, therefore producing higher sensitivity. However, the selectivity will be lower, and thus the LOD may increase or decrease, depending upon the complexity of the sample. Table 5-4a shows that the LOD for different lengths of GC columns are similar. However, dramatic differences are observed in the slopes of the calibration curves for the different lengths of GC columns in Table 5-4b. The ratio of the slopes of the calibration curves for the 30 m GC column divided by the 10 m GC column for standard PGE_2 utilizing SIM is 0.22 and for SRM is 0.29. This indicates that analysis with the 30 m GC column is approximately 26% as sensitive as that with the 10 m GC column. In addition, peak areas for the 500 pg of $^2H_4$-PGE_2 internal standard utilizing a 10 m GC column are approximately four times larger than for a 30 m GC column. Therefore, these results suggest that greater than 75% of the derivatized PGE_2 is lost somewhere along the 30 m GC column. This may occur due to adsorption on the GC column or decomposition of the derivatized PGE_2 on the column. Similar observations were made in an earlier report with a 30 m GC column (112). The researchers reported that after repeated analyses of derviatized PG samples on a 30 m GC column, substantial loss of particular PGs (e.g.,

PGE_2, but not $PGF_{2\alpha}$) occured. Consequently, PGE_2 would have less time and length of column to interact with the stationary phase when shorter GC column lengths (e.g., 3 m or 10 m) are utilized.

The chromatograms in Figure 5-9 show the obvious time differential between the three lengths of GC column. Analysis of PGE_2 with a 30 m GC column requires ~15 min., with a 10 m GC column ~5 min and with a 3 m GC column ~3.5 min. Since these chromatograms utilize the same sample preparation method, the only difference in the time of analysis is based on the length of the GC column. Furthermore, the 30 m GC column requires higher, elevated temperatures for elution of PGE_2 in urine (~305°C), than a 10 m (~260°C) or 3 m (~240°C) GC column. This elevated temperature corresponds to an additional time increase required to cool the oven to the initial starting temperature of 100°C. If we compare the total GC analysis time, an analysis utilizing a 30 m GC column (Figure 5-9a) would require ~25 min before injecting the next sample compared to ~12 min for a 10 m GC column. This corresponds to the analysis of 20 samples a day with a 30 m GC column with these conditions compared to 40 samples a day with a 10 m GC column. Thus, for a slight loss in selectivity with a 10 m GC column, twice the number of samples can be quantitated in a day for PGE_2 in urine.

A summary similar to the one presented earlier for sample preparation (Figure 5-8) is shown in Figure 5-10 for sample introduction. This figure indicates graphically the relative trade-offs between the selectivity and time of analysis for the three different GC columns. Figure 5-10a shows that a 30 m column possesses the greatest selectivity; however, Figure 5-10b displays the fact that this same length of GC column

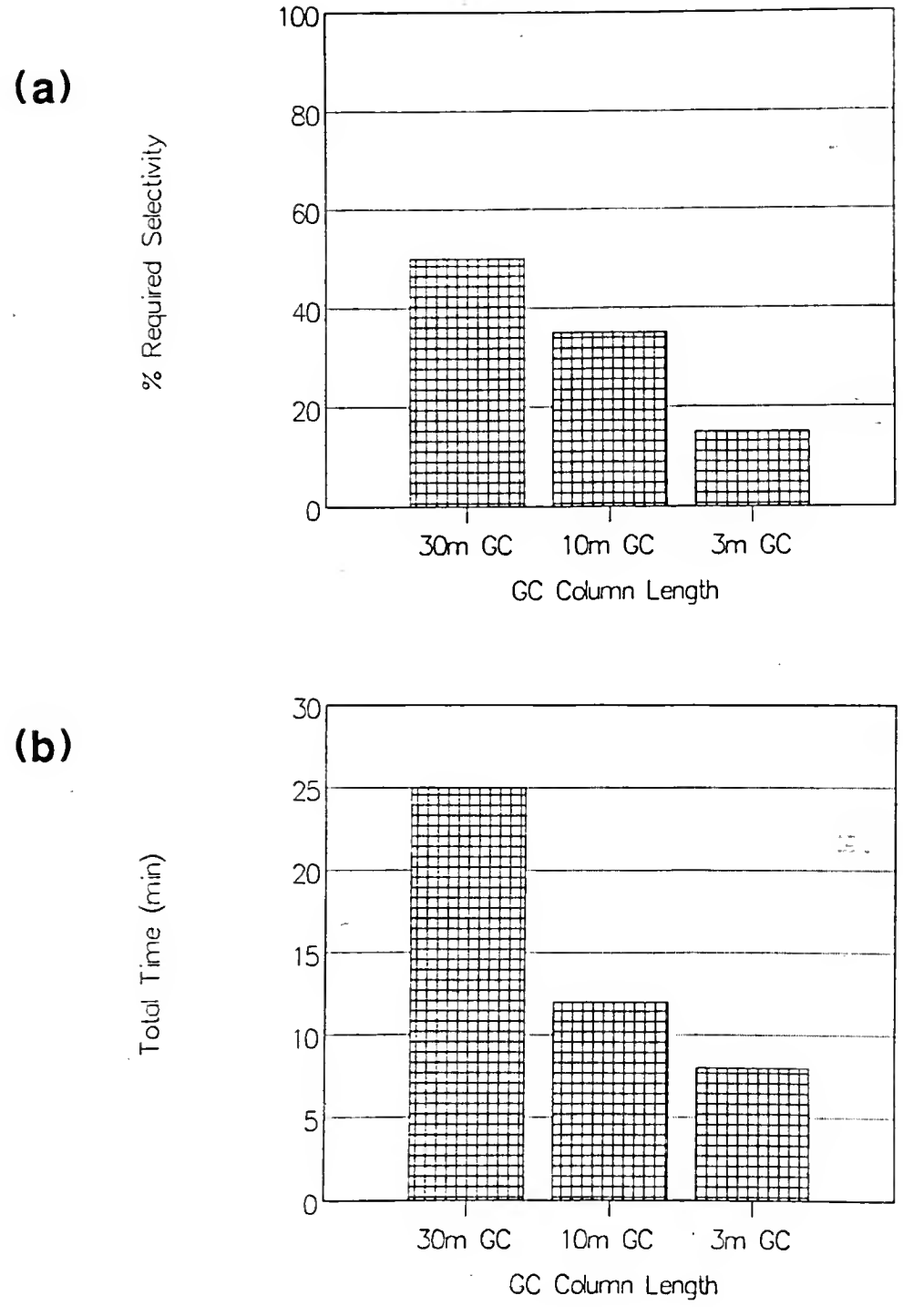


Figure 5-10: Summary of the relative differences in GC column lengths for: (a) Selectivity (b) Total time between GC injections

has a total analysis time twice as long as a 10 m GC column. Finally, the three GC columns do not possess enough selectivity without sample preparation and a detection scheme to reach the required selectivity line on the figure.

Mass Spectrometric Analysis: SIM vs SRM

The advantages of using SRM for mixture analysis have been pointed out earlier in Chapter 1. Utilization of SRM reduces the chemical noise inherent in analysis of complex biological matrices (94,95). Figure 5-11 compares the SIM chromatogram to the SRM chromatogram of endogenous PGE_2 in urine employing C18 extraction with a 30 m GC column. The reduction of the chemical interference signal by SRM (Figure 5-11b) is apparent. This allows for more reliable quantitation of PGE_2 in urine. In addition, with SRM, in this case, the use of $2H_4$-PGE_2 is not required as a "marker compound" to mark the retention time of the trace level of endogenous PGE_2. The additional selectivity of SRM is even more apparent in Figure 5-12. This analysis method employs C18 and IA for sample preparation with a 3 m GC column. In Figure 5-12a with SIM, the endogenous level of PGE_2 is only slightly visible above the chemical interferents present. However, Figure 5-12b demonstrates the advantage of SRM for this analysis. The chemical interferences are completely eliminated, allowing for accurate, reliable quantitation of endogenous levels of PGE_2 in urine.

The sensitivity of SIM compared to SRM was discussed earlier in Chapter 3. The sensitivity for SIM should be greater than SRM when the analyte signal is efficiently separated from the chemical interference signal present in a chromatogram. In Table 5-4b, the slope of the

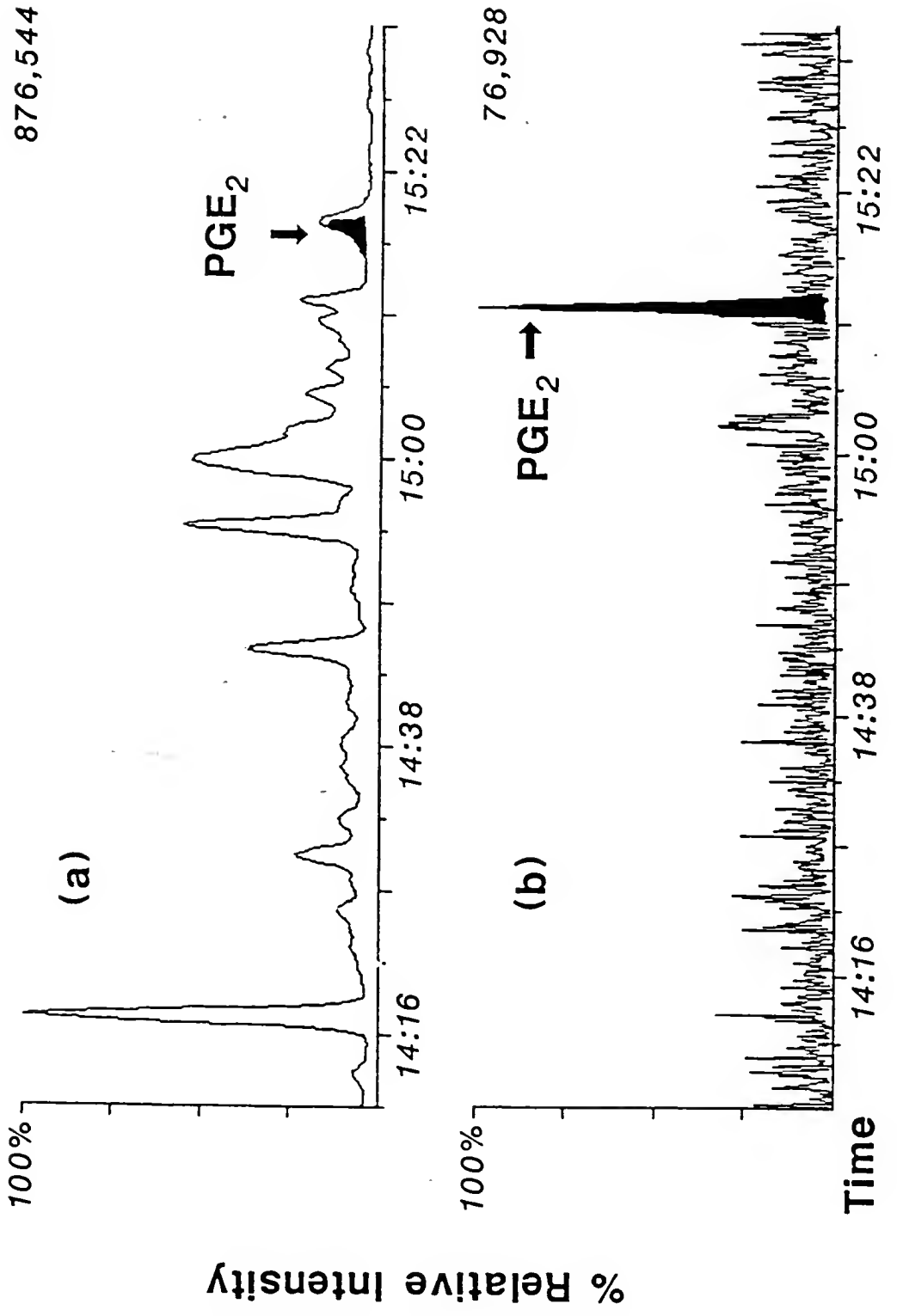


Figure 5-11: 30 m GC column chromatograms of endogenous PGE_2 in urine extracted with a C18 column utilizing: (a) SIM (b) SRM

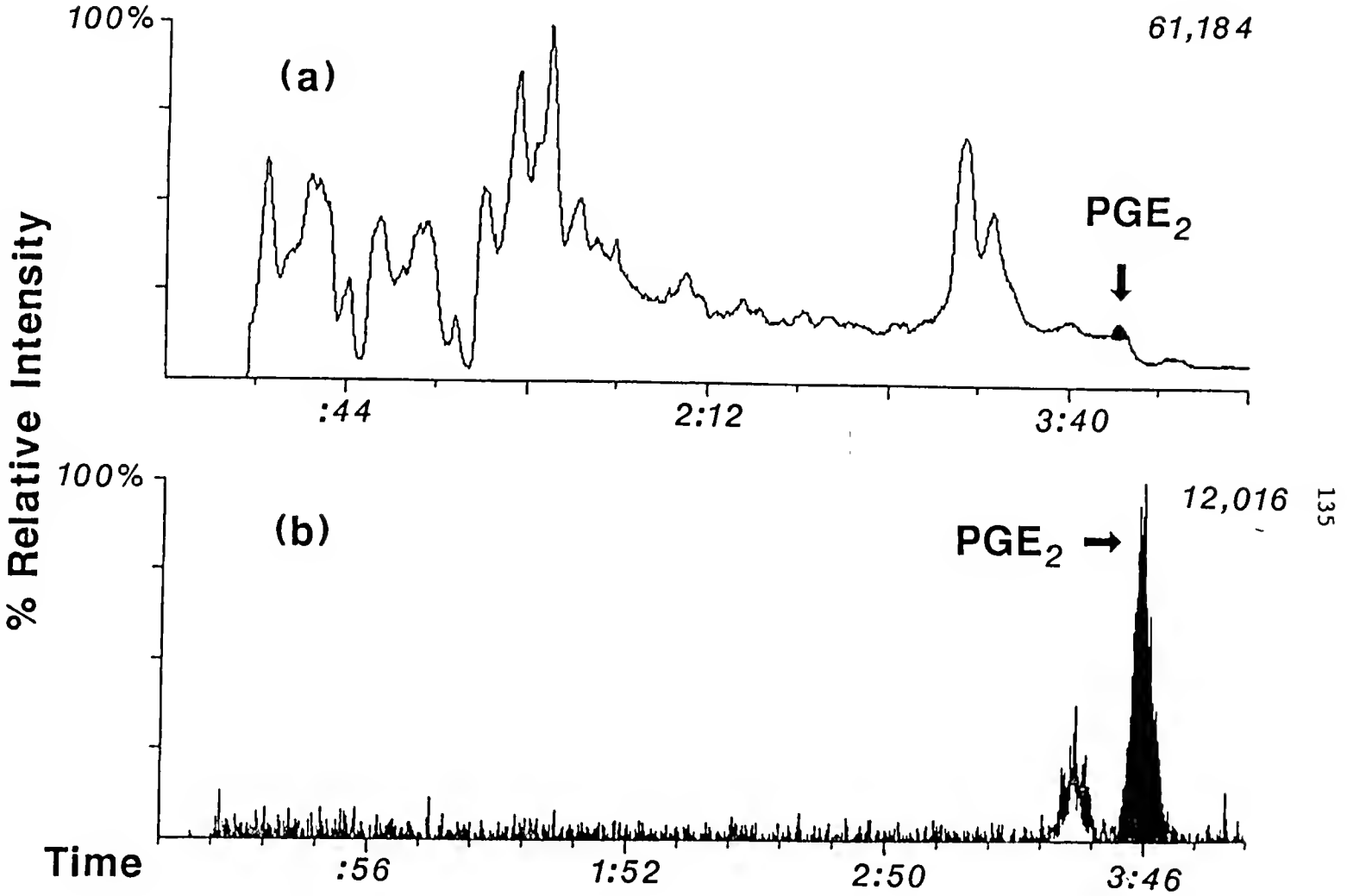


Figure 5-12: 3 m GC column chromatograms of endogenous PGE_2 in urine extracted with C18 and IA columns utilizing: (a) SIM (b) SRM

calibration curve for SIM and SRM utilizing a 3 m GC column may be compared to determine the relative difference in sensitivity between SIM and SRM. The ratio of the SRM slope to that of the SIM slope is 0.035. This indicates that the sensitivity of SIM is 30 times greater than SRM for derivatized standard PGE_2. The lower sensitivity of SRM is expected due to the inefficiency of the CAD conversion of the parent ion to the daughter ion of interest. This was investigated and discussed in detail in Chapter 3 for PGE_2, resulting in an overall CAD efficiency of approximately 12% for the selected-reaction chosen. In addition, for SRM the second mass analyzer (Q3) is used as a mass filter rather than an RF only quadrupole, thus, a lower transmission efficiency is expected (typically 10%). However, the selectivity gained by the parent-daughter reaction reduces the chemical noise to a greater extent than the analytical signal in a sample matrix, often compensating for the lost sensitivity.

The differences in the LOD for different sample preparations and GC column lengths utilizing SIM and SRM can be found in Table 5-4a. The lowest limits of detection (20 pg) found were with methods A, B, C, and G, which employed C18 and IA sample preparation with a 10 m GC column using SIM. The LODs for all the analytical schemes vary by only 15 pg, except for method H which employed C18 and IA extraction with a 10 m GC column using SRM.

The time for analysis by SIM or SRM is essentially the same; however, as was pointed out earlier in Chapter 3, optimization of the selected reactions to be employed in the analysis is essential. This optimization requires an additional amount of time in the beginning of an

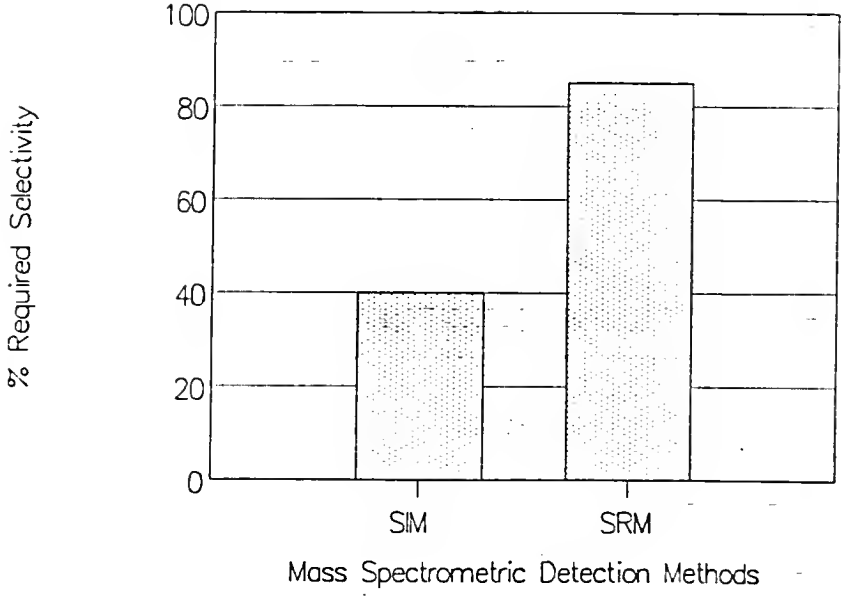


Figure 5-13: Summary of the relative differences in selectivity for SIM and SRM.

analysis, but once accomplished can give an enhancement in selectivity which is worthwhile. More importantly, the use of SRM rather than SIM may provide for shorter analysis times due to possible reduction of extensive sample preparation and chromatographic separation.

Figure 5-13 graphically summarizes the relative selectivity differences observed between SIM and SRM for analysis of PGE_2 in urine. This graph is similar to the previous figures; however, the time of analysis for SRM and SIM (essentially the same) is not shown. Therefore, SRM possesses greater selectivity than SIM and requires the same amount of time.

Conclusions

The basic steps in the analytical scheme for the determination of endogenous PGE_2 in urine have been systematically investigated and evaluated for their selectivity, sensitivity and time of analysis. The selectivity of the techniques investigated have been summarized throughout the chapter. Comparing the relative selectivity of the sample preparation, GC column lengths, and mass spectrometric (MS) detection methods (Figure 5-14a) the various parameters individually lack the necessary selectivity to analyze PGE_2 in urine. However, by combining these methods into a logical analytical scheme the selectivity required for the analysis may be achieved. Figure 5-14b shows the selectivity of various combinations of sample preparations, GC column lengths and MS detection methods. In method C, the use of C18 and IA purification with a 30 m GC column utilizing SIM produces the selectivity required to analyze endogenous levels of PGE_2. Method K uses the same sample

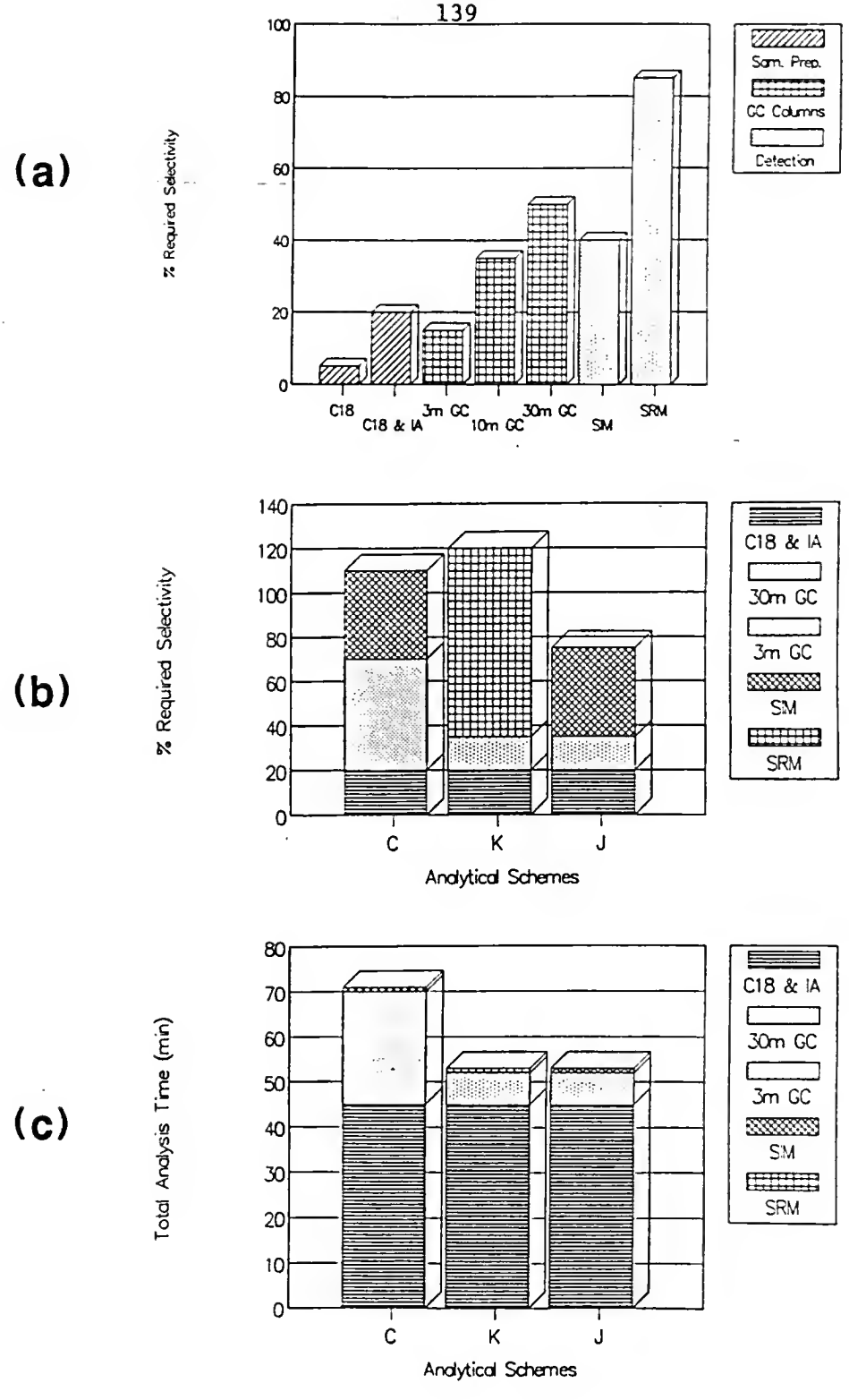


Figure 5-14: Summary of the relative differences in:
(a) Selectivity for various parameters
(b) Selectivity for various analytical schemes
(c) Total time of analysis for various analytical schemes

preparation as A, but utilizes a shorter 3 m GC column with SRM. This combination of parameters also achieves the necessary selectivity. However in method J, using the same sample preparation and GC column, but with SIM rather than SRM, the needed selectivity is not achieved. Therefore, many combinations of parameters may be explored to achieve the required selectivity for a particular analysis.

Sensitivity must also be considered in an analytical scheme. In the case of the analysis of PGE_2 in urine, all of the methods utilizing SIM have greater sensitivity than the methods employing SRM. In addition, the results in this chapter demonstrate the advantage of utilizing shorter GC column lengths to obtain greater sensitivity. However, if urine matrix components interfere with the determination of endogenous levels of PGE_2, then both sensitivity and selectivity are critical. Therefore, once combinations of parameters are found which achieve the needed selectivity for an analysis, the particular method with the greater sensitivity can be selected. In this case, the 10 m GC column with SIM using C18 and IA for sample preparation could be utilized.

The time of analysis for particular methods was summarized for sample preparation and GC column lengths earlier in this chapter. The trade-offs between selectivity, sensitivity, and the total analysis time can be compared by examining the chromatograms throughout the chapter. Overall higher selectivity (and therefore lower LODs) for sample preparation and introduction methods require longer times of analysis. The total analysis time for methods C, K and J is shown in Figure 5-14c. Comparing this figure with Figure 5-14b for selectivity, the obvious trade-offs between total analysis time and selectivity for these

particular methods can be observed. As was discussed earlier, for a slight decrease in selectivity, a large decrease in the analysis time can be achieved. For example, the selectivity and sensitivity difference is quite small when comparing method C to method K; however, method K utilizing a 3 m GC column and SRM is 20 min faster per sample than method C with a 30 m GC column with SIM or SRM. This corresponds to analysis of approximately 3 to 4 times as many samples per day for a slight decrease in selectivity which does not affect the quantitation of PGE_2 in urine. Thus, trade-offs are necessary between the different steps in an analysis scheme to achieve the optimum technique.

CHAPTER 6

EVALUATION OF SOLID-PHASE EXTRACTION PROBE/MS/MS FOR THE ANALYSIS OF ENDOGENOUS PROSTAGLANDIN E_2 IN URINE

Introduction

In Chapter 5, the use of C18 and immunoaffinity (IA) purification coupled to a short 3 m GC column with selected-reaction monitoring (SRM) was shown to be a rapid analysis method with the required selectivity and sensitivity for the determination of PGE_2 in urine. However, the derivatization procedure required for GC sample introduction is both lengthy and tedious. If a sample introduction method which did not require such derivatization were employed, total analysis times could be reduced dramatically. Note, however, that it would still be attractive to retain the PFB derivatization, since it provides the opportunity to utilize the advantage of increased sensitivity and selectivity inherent in EC-NCI. Although prostaglandins (PGs) cannot be separated by GC with only PFB derivatization, it would be possible to employ a less selective sample introduction technique, such as conventional solids prode MS or direct chemical ionization probe (DCI). Unfortunately, the total cycle time for probe sample introduction is actually longer than for short GC column introduction discussed in Chapter 5. Furthermore, the fact that essentially all mixture components desorb into the ion source at nearly the same time increases the probability of quenching of the electron-

capture process. This ionization interference leads to decreased ion signals and poor limits of detection, especially in samples containing complex matrices in which an enormous number of compounds may have been derivatized which can undergo electron-capture. The objective of this study was to evaluate the capability of IA purification coupled to the sample introduction techniques of solids probe and DCI with SRM to analyze endogenous levels of PGE_2 in urine.

Experimental

Sample Preparation

The prostaglandins and reagents were identical to those described in Chapter 2. Urine samples (identical to the three in Chapter 5) and standards for a calibration curve were prepared by the same method as was explained in Chapter 5. Note that a constant amount of $2H_4$-PGE_2 was added as an internal standard to the urine before extraction. In these experiments, C18 and IA purification was utilized for all the urine samples.

Derivatization

The purified samples were evaporated and then derivatized to the PFB ester according to the procedure in Chapter 2. The methoximation and silylation steps of the procedure were not employed for this study. The amount of PFB esterification reagents used to derivatize the urine samples was tripled for the same reason as was explained in Chapter 5. After evaporation of the excess PFB esterification reagents, the derivatized samples were diluted by adding 60 μL of ethyl acetate to the urine samples

and 50 μL to the standards for the calibration curve. One-microliter injections containing 500 pg of $2H_4$-PGE_2 were either directly placed in sample vials for the solids probe or coated onto the wire loop of the DCI probe.

Instrumental Conditions

A Finnigan MAT triple stage quadrupole (TSQ45) gas chromatograph/ tandem mass spectrometer was used for these studies. Mass spectrometry conditions were: interface and transfer line temperature 300°C, ionizer temperature 190°C, electron energy 100 eV and emission current 0.3 mA. Electron-capture negative chemical ionization (EC-NCI) was carried out with methane at an ionizer pressure of 0.50 torr. Argon was employed as the collision gas at a pressure of 1.0 mTorr; a collision energy of 25 eV was utilized for the selected-reaction monitoring studies of PGE_2. The SRM experiments employed an EM setting of 2000 V and a preamp gain of 10^8 V/A. A calibration curve was obtained for the DCI studies by analyzing standard solutions containing a constant amount of $^2H_4$-PGE_2 (25 ng) and increasing amounts of PGE_2, as listed in Table 5-2 in Chapter 5.

The quantitation of the endogenous levels of PGE_2 in urine was accomplished by selected-reaction monitoring (SRM) of $351^- \rightarrow 271^-$ for PGE_2 and $355^- \rightarrow 275^-$ for the internal standard, $^2H_4$-PGE_2. A baseline was chosen visually on the solids probe or DCI probe trace and the areas for PGE_2 and $^2H_4$-PGE_2 calculated by the INCOS computer system for the calibration curve and the extracted urine samples. The area of PGE_2 divided by the area of $^2H_4$-PGE_2 in the standards gives a ratio which is used in the calibration curve. The amount of PGE_2 in each urine sample was calculated according

to the method described earlier in Chapter 5.

The solids probe analysis was performed by placing the diluted urine sample or standard into a small glass vial and evaporating the ethyl acetate in air. The probe temperature was initially set at 50°C and then increased at 60°C/min to 300°C for the standards, urine blank and urine samples analyzed. The DCI analysis was performed by coating the wire loop of the Finnigan direct exposure probe with the sample and allowing the ethyl acetate to evaporate in air. The heating current (initially 0 mA with the probe at room temperature) was increased at 10 mA/s (600°C/min) for 1 min.

Solids Probe Analysis

EC-NCI and Daughter Spectra of Standard PGE_2

The EC-NCI mass spectrum for standard PGE_2 with no derivatization is shown in Figure 6-1a. This mass spectrum of 1 μg of PGE_2 is dominated by ions corresponding to the loss of one (334^-) and two (316^-) water molecules from the $M^{-\cdot}$ ion. The $M^{-\cdot}$ ion of PGE_2 (351^-) has a low relative abundance of only 0.5% compared to the intense ions observed from the loss of one and two water molecules. The large relative abundance of the 334^- and 316^- ions suggests that dehydration of the PGE_2 molecules may occur before the ionization process, as they are desorbed from the glass sample vial as the probe is heated. In addition, positive chemical ionization (PCI) was performed and evaluated. The results from thse experiments show that without derivatization, the PCI mass spectrum has a much lower sensitivity than that of EC-NCI.

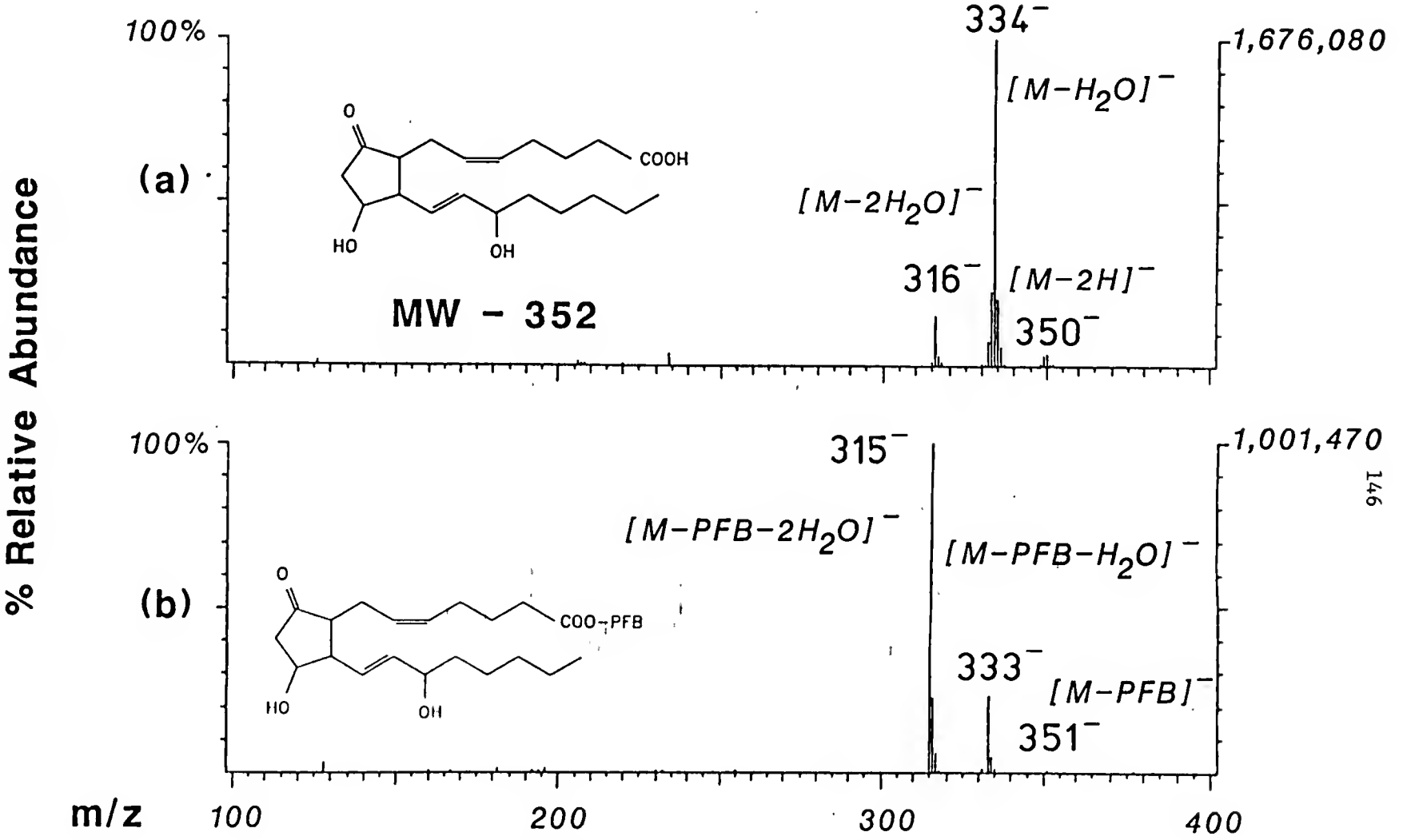


Figure 6-1: Solids probe electron-capture negative chemical ionization mass spectrum of:
(a) Underivatized PGE_2 standard (1μg)
(b) PFB ester derivative of PGE_2 (5 ng).

The PFB derivative of PGE_2 was prepared and evaluated to compare the sensitivity utilizing this abbreviated derivatization procedure to that of the underivatized standard. Figure 6-1b shows the EC-NCI mass spectrum of 5 ng of the PFB ester derivative of PGE_2. This mass spectrum is similar to Figure 6-1a of the underivatized PGE_2; however, the most abundant ion is the $[M\text{-}PFB\text{-}2H_2O]^-$ ion rather than the $[M\text{-}H_2O]^-$ ion. The $[M\text{-}PFB\text{-}H_2O]^-$ ion is also prominent in Figure 6-1b, with other less intense ions (< 5%) present in the mass spectrum. Again, these ions may reflect thermal dehydration as the PFB derivatized PGE_2 is vaporized off the probe. The 351^- ion corresponds to the $[M\text{-}PFB]^-$ ion produced by dissociation electron capture, and has a low intensity compared to the other ions in the spectrum. Comparing the absolute counts observed for the base peaks in Figure 6-1a with Figure 6-1b, the underivatized PGE_2 signal is 1.7 times larger than that for the PFB derivatized PGE_2 standard; however, the underivatized PGE_2 sample had a concentration 200 times greater than the PFB-derivatived PGE_2. Thus, the sensitivity for the PFB-derivatized PGE_2 is approximately 100 times greater than that of the underivatized standard.

The extremely low relative abundance of the $[M\text{-}PFB]^-$ ion in the EC-NCI mass spectrum, subsequently produces a low intensity daughter spectrum of the $[M\text{-}PFB]^-$ ion (Figure 6-2). A daughter spectrum with many low intensity fragment ions is shown in Figure 6-2. The most abundant ion in the spectrum is the 271^- ion corresponding to $[M\text{-}PFB\text{-}2H_2O\text{-}CO_2]^-$. Subsequently, this ion was chosen for selected-reaction monitoring of PGE_2 in urine. The other prominent daughter ions are listed in Table 6-1 with the % relative abundance and probable ion assignment. Note that the

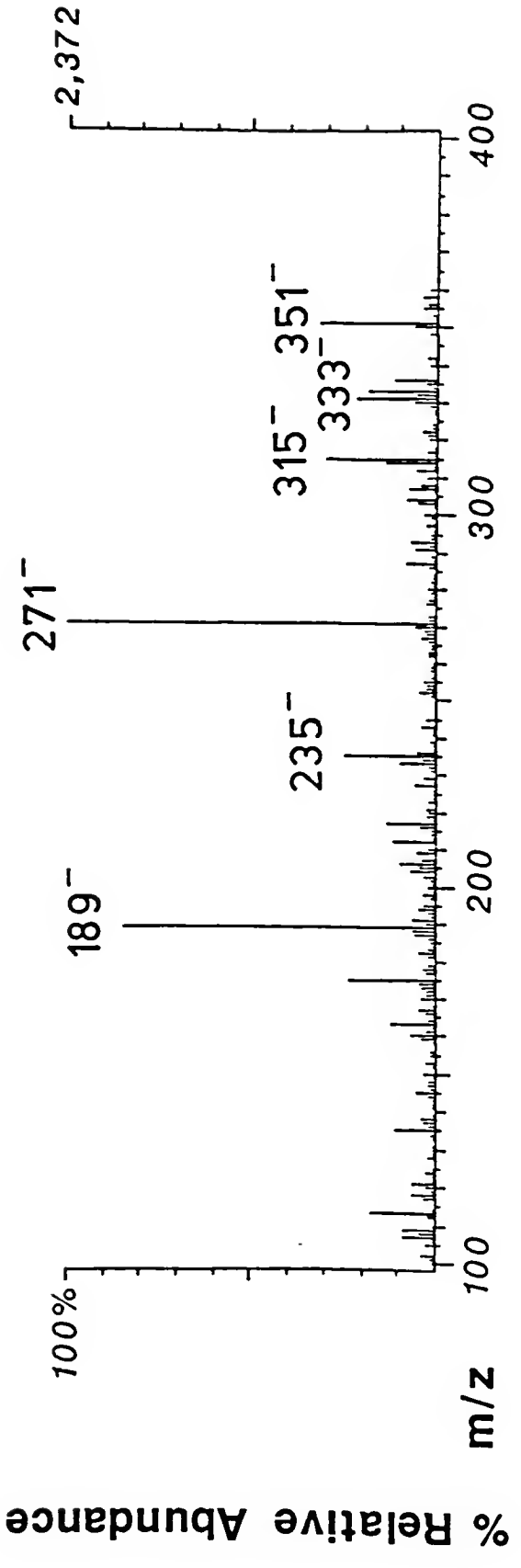


Figure 6-2: Solids probe daughter ion spectrum of the $[M\text{-}PFB]^-$ ion of the PFB ester derivative of PGE_2 (5 ng) at 1.0 mTorr of argon and 25 eV collision energy.

Table 6-1: Daughter Ions of $[M\text{-}PFB]^-$ (P^-) of PGE_2 PFB Ester Analyzed by Direct Solids Probe

Ion Assignment	m/z	%RA[a]
$[P]^-$	351	32
$[P\text{-}H_2O]^-$	333	23
$[P\text{-}2H_2O]^-$	315	31
$[P\text{-}2H_2O\text{-}CO_2]^-$	271	100
$[P\text{-}2H_2O\text{-}CO_2\text{-}C_2H_2]^-$	235	28
$[P\text{-}2H_2O\text{-}CO_2\text{-}C_5H_6O]^-$ or $[P\text{-}2H_2O\text{-}CO_2\text{-}C_6H_{10}]^-$	189	85

[a] % Relative abundance at a collision gas pressure of 1.0 mTorr argon and collision energy of 25 eV.

$[M\text{-}PFB\text{-}H_2O]^-$ ion in the EC-NCI mass spectrum could have been chosen for MS/MS, with an increase in sensitivity of approximately 100.

Study of Endogenous Levels of PGE_2 in Urine

Figure 6-3 shows the SRM traces of a 350 pg PGE_2 standard, a urine blank (containing endogenous levels of PGE_2 with no $^2H_4$-PGE_2 added) and a urine sample containing 500 pg of $^2H_4$-PGE_2. The urine blank and sample were prepared by essentially the same method with C18 and IA purification then derivatized to the PFB ester of PGE_2. This experiment was performed with a temperature ramp of 60°C/min, as described in the experimental section of this chapter. The trace for the PGE_2 standard (Figure 6-3a) shows one distinct peak with a low level of noise. Figure 6-3b shows the chromatogram of the urine blank with the same conditions as the standard in Figure 6-3a. A distinct peak for endogenous PGE_2 is noticed, but in this case the noise level has increased drastically compared to the relative intensity of the PGE_2 peak. The peak area of the PGE_2 standard in Figure 6-3a is 5 times greater than the peak area of the endogenous level of PGE_2 in the urine blank (Figure 6-3b). Results from Chapter 5 indicate that endogenous levels of PGE_2 in urine are approximately 300 to 350 pg/mL. Therefore, the peak area for the urine blank should be approximately the same as that of the 350 pg PGE_2 standard in Figure 6-3a. This suggests that PGE_2 may have been lost during the sample preparation procedure or the remaining urine matrix components in the extracted sample interfere with the efficient ionization of the $[M\text{-}PFB]^-$ ion. This second possible explanation is reffered to as quenching and may

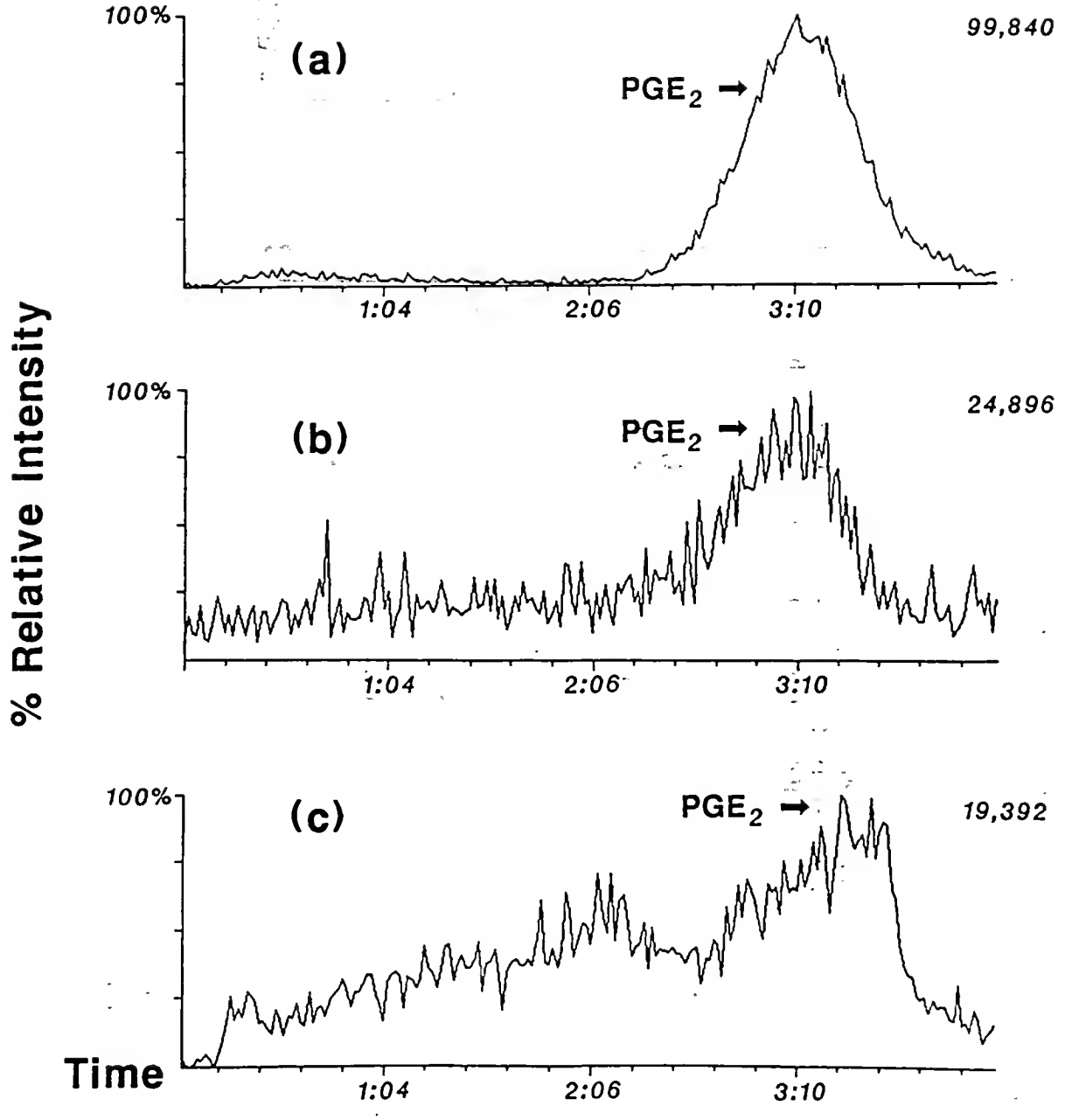


Figure 6-3: Solids probe SRM trace of a PFB derivatized:
(a) 350 pg PGE_2 standard
(b) Extracted urine blank
(c) Extracted urine sample containing 500 pg of $^2H_4$-PGE_2 internal standard

occur in the analysis of biological samples when EC-NCI is utilized, without extensive chromatographic clean-up (111).

The trace for a urine sample which contained an additional 500 pg of $^{2}H_{4}$-PGE_{2} which was not present in the urine blank (Figure 6-3b) is shown in Figure 6-3c. The peak shape for PGE_{2} is not as distinct as in Figures 6-3a and 6-3b and the signal to noise ratio has decreased considerably from the 350 pg standard in Figure 6-3c. Comparing the PGE_{2} standard in Figure 6-3a to the urine sample, the peak area is approximately 7 times lower than that calculated for the PGE_{2} standard. In addition, the peak area of the urine blank (Figure 6-3b) is 1.5 times greater than the peak area of the urine sample containing the $^{2}H_{4}$-PGE_{2}. These results suggest that the urine matrix components in the urine samples leads to the quenching effect discussed above.

Slower temperature ramps were evaluated to determine if PGE_{2} could possibly be separated from the urine matrix interferences present in the extract. However, the quenching effect noted in the figures above was still a prominent problem. In addition, calculation of an accurate peak area was extremely difficult due to the low signal to noise ratio observed for urine samples.

Figure 6-3 indicates the difficulty in determination of PGE_{2} in urine by utilizing the solids probe for sample introduction. This technique lacks the selectivity and sensitivity required for the analysis of endogenous levels of PGE_{2}. The rapid process of heating the probe and vaporization of the sample from the vial results in a lack of separation of the PGE_{2} from the urine matrix components present even after IA purification. In addition, the remaining urine matrix components

apparently lead to quenching of the $[M-PFB]^-$ ion during the ionization process, resulting in inefficient SRM. Therefore, after examining these results and experimenting further with different temperature ramps, the solids probe/MS/MS method was not employed for quantitation of PGE_2 in urine.

Direct Chemical Ionization Analysis

Concept of Direct Chemical Ionization (DCI)

The technique of DCI, also called "in beam" or "desorption chemical ionization", mass spectrometry was first introduced by Baldwin and McLafferty (113) and appears to be useful in the analysis of medium-molecular-weight polar compounds (114,115). Typically, the technique involves coating a wire probe with a sample which is then directly inserted into the ion plasma in the chemical ionization source. The probe is usually heated with an electric current to temperatures in excess of 1000°C. In most cases, polar organics are volatilized intact from the probe tip at temperatures much lower than this (100°C to 300°C). Recently, this technique has been utilized for both positive and negative ammonia DCI mass spectrometry of a number of PGs, without prior derivatization (80,116). This report demonstrated the advantages of DCI mass spectrometry in analyzing standard prostaglandins without derivatization and the mechanistic aspects of the gas-phase chemistry involved. However, only PG standards were investigated, and at levels (50 ng or more) well above endogenous levels of PGE_2 in urine. Therefore, this rapid sample introduction technique with a short derivatization procedure was evaluated for determining endogenous PGE_2 in urine.

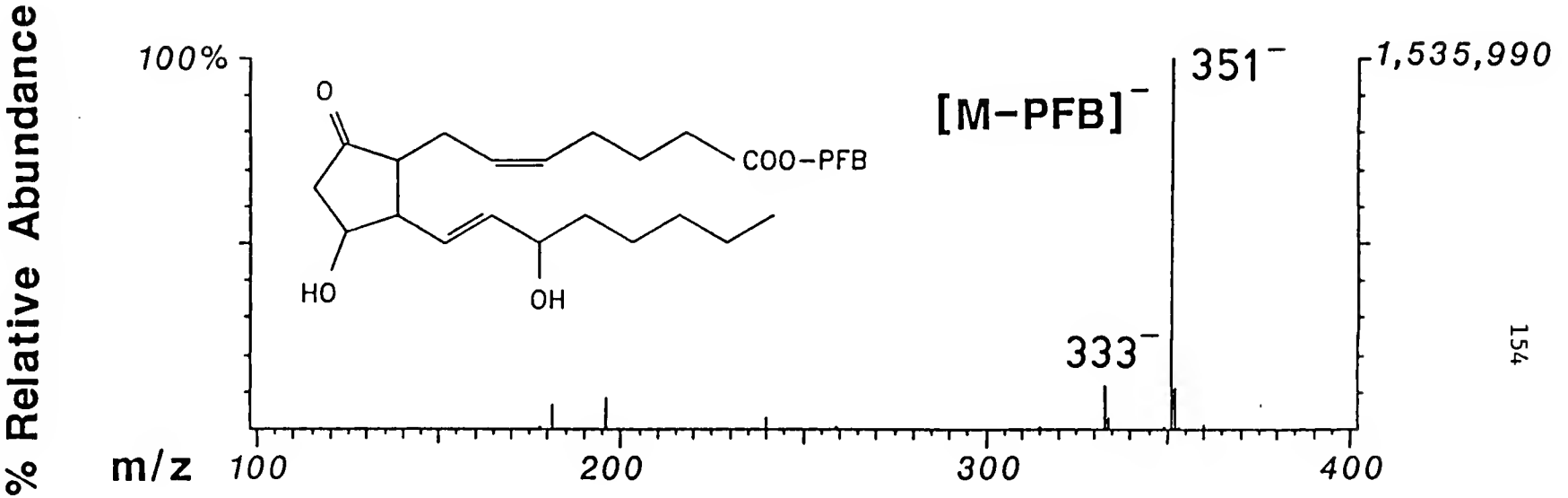


Figure 6-4: Direct chemical ionization electron-capture negative chemical ionization mass spectrum of PFB derivatized PGE_2 standard.

EC-NCI and Daughter Spectra of Standard PGE_2

Figure 6-4 shows the EC-NCI mass spectrum of 500 pg of a PFB derivatized PGE_2 standard. This spectrum differs from that of the PFB-derivatized PGE_2 using the solids probe (Figure 6-1b) in that the most prominent ion is the $[M-PFB]^-$ carboxylate anion (351^-), rather than the ions corresponding to a loss of one or two water molecules. The importance of acquiring an intense parent ion ($[M-PFB]^-$) was discussed earlier. In the case of DCI, since the $[M-PFB]^-$ carboxylate anion is the most abundant ion in the EC-NCI mass spectrum, the sensitivity should be enhanced compared to the solids probe. Differences in the EC-NCI mass spectra of PFB derivatized PGE_2 standard using solids probe and DCI may be attributed to the binding of the PG sample to the glass vial used in solids probe analysis, as was discussed earlier in this chapter. The loss of one and two water molecules from the $[M-PFB]^-$ are present in the mass spectrum as well as a few low intensity "backbone" fragment ions. The absolute counts for the $[M-PFB]^-$ ion of a 500 pg sample of PFB derivatized PGE_2 using DCI was 1.5 times greater than the counts for the $[M-PFB-2H_2O]^-$ ion of a 5 ng PFB-derivatized PGE_2 standard using the solids probe, corresponding to a 15 times higher sensitivity for DCI. Therefore, this method should provide adequate sensitivity for the analysis of PGE_2 and may be comparable to that found for a 3 m GC column utilizing SRM in Chapter 5.

Daughter ion spectra of the $[M-PFB]^-$ ion (351^-) under different CAD conditions are shown in Figure 6-5a and Figure 6-5b. The first (Figure 6-5a) daughter spectrum was obtained with a collision energy of 28 eV and a collision gas pressure of 1.5 mTorr of argon. Figure 6-5b was obtained

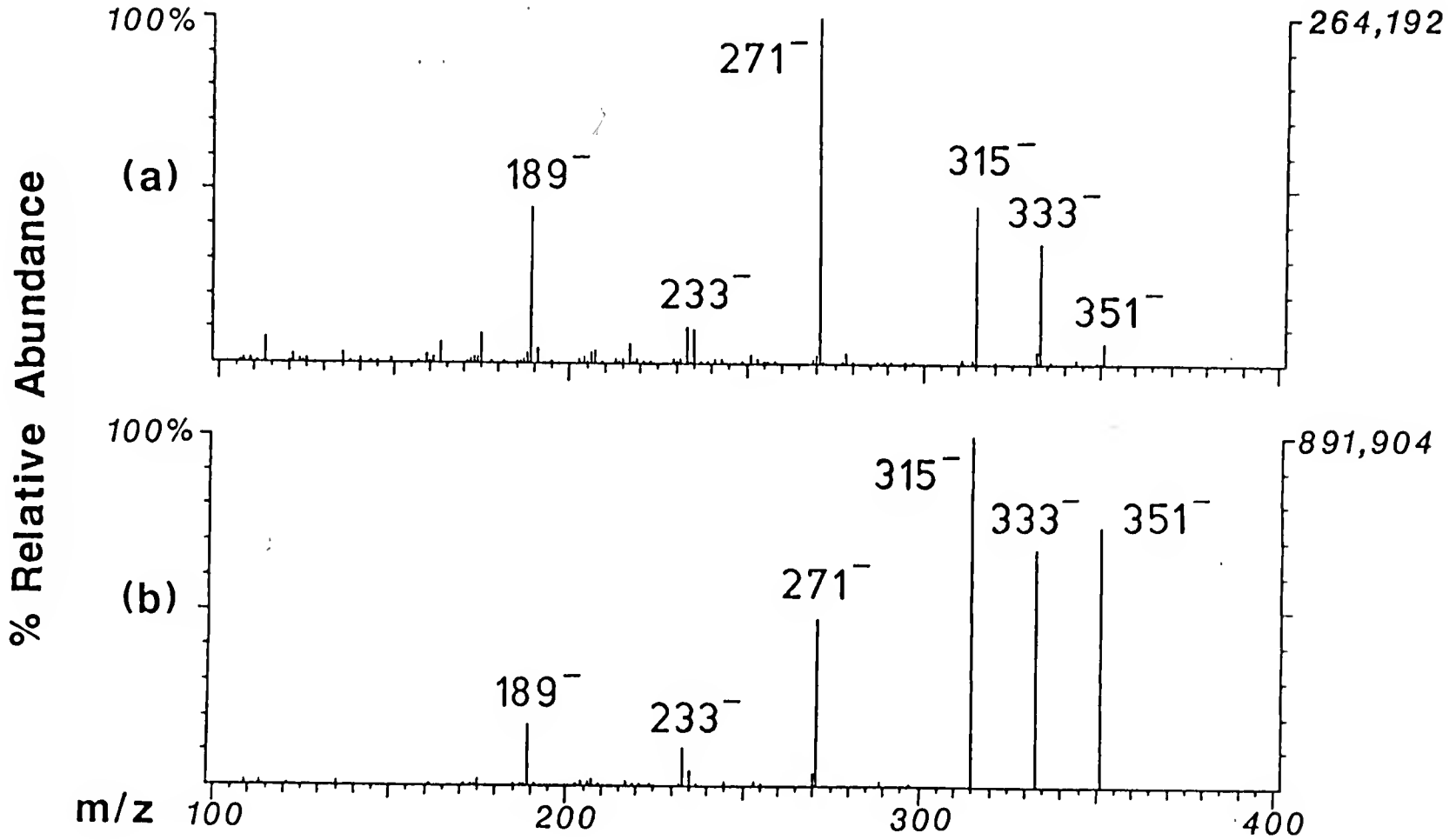


Figure 6-5: Direct chemical ionization daughter ion spectra of the $[M\text{-}PFB]^-$ ion of the PFB ester derivative of PGE_2 at:
(a) 1.5 mTorr of argon and 28 eV
(b) 1.0 mTorr of argon and 25 eV

Table 6-2: Daughter Ions of $[M\text{-}PFB]^-$ (P^-) of PGE_2 PFB Ester Analyzed by Direct Chemical Ionization (DCI)

Ion Assignment	m/z	%RA[a]	%RA[b]
$[P]^-$	351	8	77
$[P\text{-}H_2O]^-$	333	39	69
$[P\text{-}2H_2O]^-$	315	47	100
$[P\text{-}2H_2O\text{-}CO_2]^-$	271	100	49
$[P\text{-}2H_2O\text{-}CO_2\text{-}C_2H_4]^-$ or $[P\text{-}2H_2O\text{-}CO_2\text{-}CO]^-$	233	12	13
$[P\text{-}2H_2O\text{-}CO_2\text{-}C_5H_6O]^-$ or $[P\text{-}2H_2O\text{-}CO_2\text{-}C_6H_{10}]^-$	189	45	19

[a] % Relative abundance at a collision gas pressure of 1.5 mTorr argon and collision energy of 28 eV.

[b] % Relative abundance at a collision gas pressure of 1.0 mTorr argon and collision energy of 25 eV.

with a collision energy of 25 eV and a collision gas pressure of 1.0 mTorr of argon. The prominent ions in both daughter spectra are the same and are listed in Table 6-2 with the % relative abundances and possible ion assignments. The fragment ions which occur at lower mass are ions that correspond to backbone-specific fragments, due to fragmentation of the carbon-hydrogen skeleton in PGE_2. Differences in the relative intensity of the ions in the two daughter spectra are noted, particularly the major ions of m/z 351, 333, 315 and 271. As was explained in Chapters 3 and 4, increasing the collision energy allows for more energetic collisions, while increasing the collision gas pressure increases the number of collisions each ion experiences. This explains the differences noted in the two daughter spectra. In addition, comparing Figure 6-5b to the daughter spectrum of the PFB-derivatized PGE_2 standard utilizing the solids probe (Figure 6-2), the spectra are similar. This is to be expected since the CAD conditions were identical in both cases. The differences in the ions relative intensity may be attributed to the use of different methods of sample introduction, and the much lower signal-to-noise ratio in Figure 6-2.

Study of Endogenous Levels of PGE_2 in Urine

Figure 6-6 shows the SRM traces of a 350 pg PGE_2 standard (Figure 6-6a), as well as a urine blank (Figure 6-6b) and a urine sample containing 500 pg of $^2H_4$-PGE_2 internal standard (Figure 6-6c) prepared by C18 and IA purification. These three samples were analyzed with DCI utilizing a 600°C/min temperature ramp as described in the experimental section. The SRM traces of of all three samples shows a distinct peak

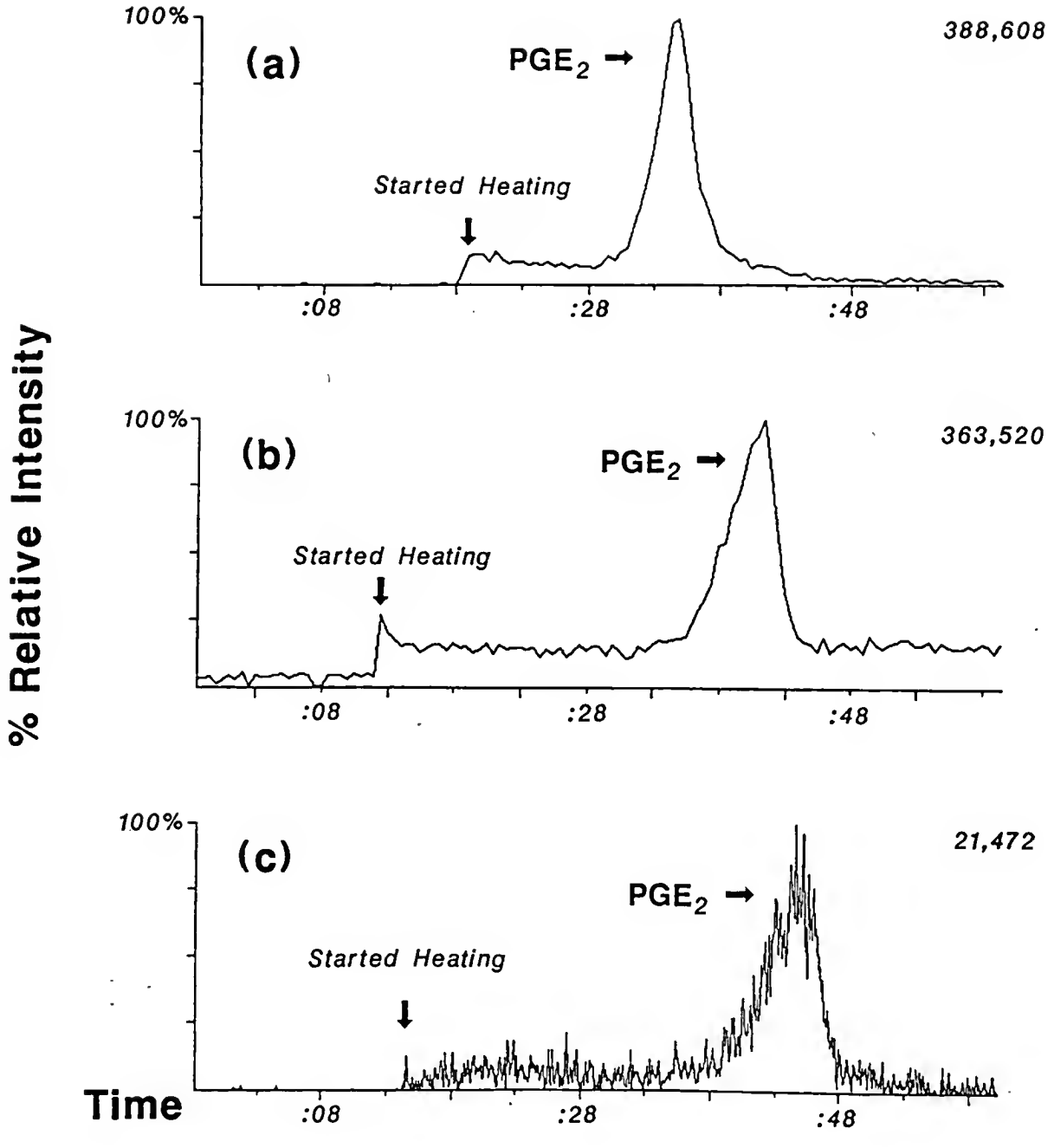


Figure 6-6: Direct chemical ionization SRM trace of a PFB derivatized:
(a) 350 pg PGE_2 standard
(b) Extracted urine blank
(c) Extracted urine sample containing 500 pg of $^2H_4$-PGE_2 internal standard

which vaporizes off the DCI probe approximately 20-30 s after the heating has started. Comparing Figure 6-6a to Figure 6-6b, the 350 pg PGE_2 standard has a peak area 6 to 7 times greater than the peak area of the endogenous PGE_2 in the urine blank. The peak area of the urine blank should be approximately the same as the 350 pg standard for the reasons described in the solids probe section. In addition, the peak area of the urine sample containing 500 pg of $^2H_4$-PGE_2 (Figure 6-6c) was 3 times lower than the peak area of the urine blank (Figure 6-6b) and 18 times lower than the peak area of the standard (Figure 6-6a), with a dramatically lower signal-to-noise ratio. The quenching effect, noted above, is more prominent for the urine sample containing the 500 pg of $^2H_4$-PGE_2.

The quantities of endogenous PGE_2 in urine obtained by utilizing DCI/MS/MS with a 600°C/min vaporization rate are listed in Table 6-3. The three urine samples were the same as reported earlier in Chapter 5. Quantitation was performed utilizing the method listed above in the experimental section. The calibration curve used to determine the amount of endogenous PGE_2 in urine is shown in Figure 6-7. The calibration curve shown demonstrates good linearity with a slope of 1.06, similar to the expected value of 1.00 for this calibration curve. Extrapolation of the x-intercept from the best fit line yields the amount of PGE_2 in 500 pg of the $^2H_4$-PGE_2 internal standard. The x-intercept obtained from this calibration curve corresponded to approximately 26 pg of PGE_2 which is 5.2% of the 500 pg of $^2H_4$-PGE_2. Results from this study establish the fact that the urine matrix components vaporize off the DCI probe at the same time as the endogenous PGE_2. The average values obtained for the three samples are approximately 3 times larger than the values found for the same urine

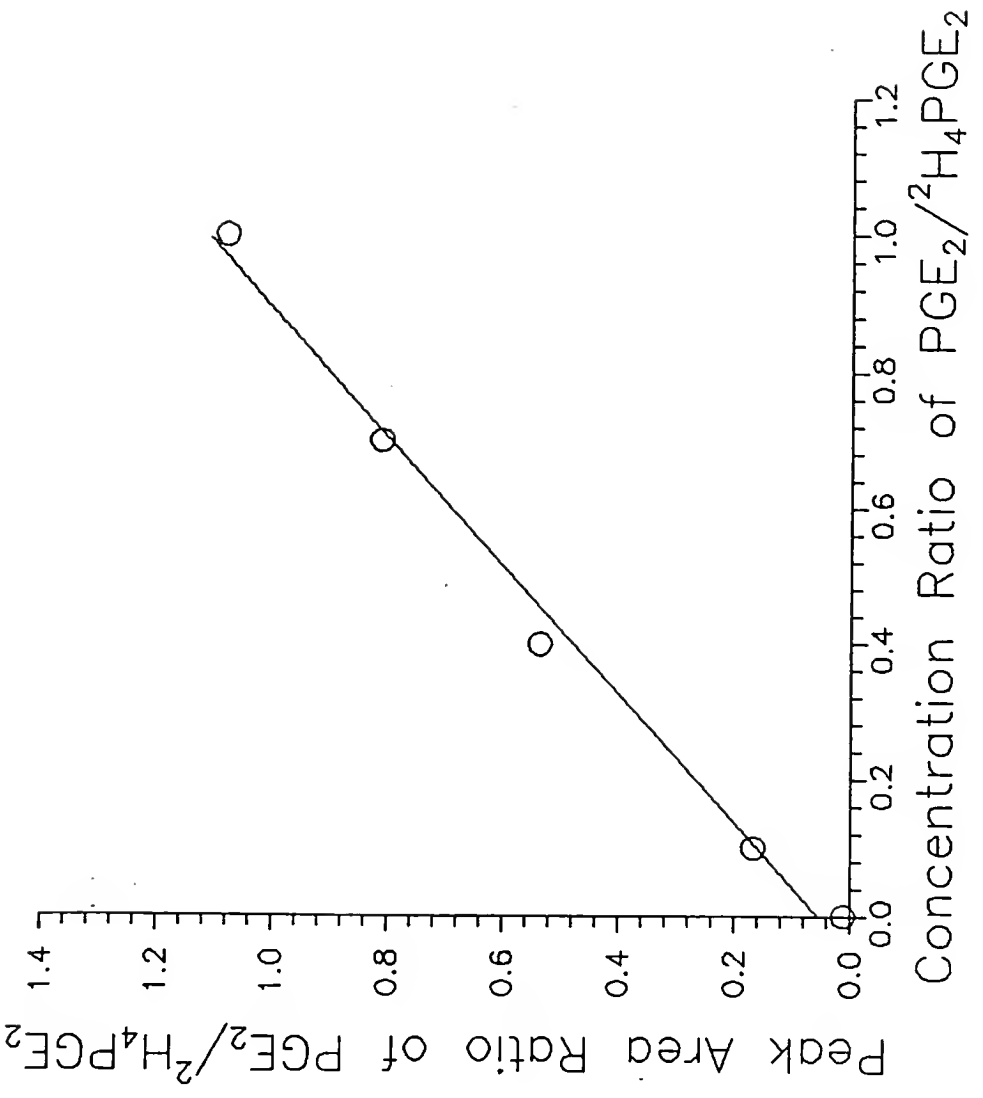


Figure 6-7: Calibration curve utilizing direct chemical ionization with SRM.

Table 6-3: Quantitation Study of Endogenous PGE_2 in Urine Utilizing Direct Chemical Ionization (DCI)/MS/MS[a]

Urine Sample #	Concentration of PGE_2 (pg/mL) DCI Injection #			Avg. of 3 Inj.	%RSD[b]
	1	2	3		
1	1063.3	1144.5	1172.1	1126.6	5.0
2	1134.0	913.8	1066.4	1038.1	10.9
3	985.7	1085.2	1191.1	1087.3	9.4

Average of Urine Samples 1 & 2: 1082.4 pg/mL

Average of 3 Urine Samples: 1084.0 pg/mL

%RSD of 3 Samples: 4.1

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[a] C18 and IA purification performed on all 3 urine samples.

[b] % Relative Standard Deviation

samples derivatized and analyzed by GC/MS/MS in Chapter 5. Therefore, the selectivity of this method is inadequate for the analysis of PGE_2 in urine. Sensitivity of the method was investigated by the same method employed in Chapter 5. The slope of the calibration curve of standard PGE_2 derivatized with PFB was 0.0023 counts/pg, approximately 4 times lower than that found for a 30 m GC column utilizing SRM. Consequently, the sensitivity may not be adequate for the analysis of endogenous PGE_2 in urine. However, examining the LOD for PGE_2 in urine, 100 to 125 pg/mL was detectable with a signal-to-noise ratio of 3. Therefore, if the selectivity of this technique could be enhanced, the LOD of DCI/MS/MS is sufficient for the determination of endogenous levels of PGE_2 in urine.

Slower temperature ramps were evaluated to determine if PGE_2 could possibly be separated from the urine matrix interferences present in the extract. The results were similar to those found for the solids probe analysis. The signal-to-noise ratios utilizing the slower temperature ramps were inadequate to reliably measure the peak area of the endogenous PGE_2 in urine.

The urine matrix components which interfere with the analysis of PGE_2 and may cause quenching are still present in the extracts due to secondary interactions (non-specific adsorption) which occur during the IA purification procedure. These interactions were discussed earlier in Chapter 2 and the consequences of these interactions are obvious from the results of the DCI/MS/MS studies. The utilization of an additional clean-up technique after the selective IA purification step was evaluated. Another IA column was employed, which had immunoaffinity for prostaglandin 6-keto$F_{1\alpha}$, after the IA purification of PGE_2. The eluent which was not

retained by the 6-keto$F_{1\alpha}$ IA column was collected and analyzed for PGE_2. This technique allowed the PGE_2 to pass unretained through this IA column, while hopefully retaining the urine matrix components which were originally non-specifically bound to the PGE_2 IA column. It was observed that the selectivity of the DCI analysis was not enhanced appreciably by this additional clean-up step.

Conclusions

The utility of solids probe/MS/MS and DCI/MS/MS for the analysis of endogenous levels of PGE_2 in urine has been evaluated in this chapter. An abbreviated derivatization method has been employed, allowing for more rapid preparation of urine samples. This advantage, coupled with the ability of DCI to vaporize samples rapidly, yields a method which reduces overall analysis times dramatically. However, as was pointed out in the introduction to this chapter, the total cycle time for probe sample introduction is actually longer than for short GC column introduction. Hence, the only savings in time results from the reduced derivatization necessary. Another drawback of the DCI probe is that the rapid vaporization of the urine sample allows a large number of urine matrix components to be vaporized into the ion source at the same time, causing quenching and creating difficult, inaccurate quantitation of endogenous levels of PGE_2, as was observed in Table 6-3. The use of DCI/MS/MS for accurate determination of PGE_2 in urine will require further investigation of more efficient and selective methods of sample preparation to eliminate the possible secondary interactions which occur in IA purification.

CHAPTER 7

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Summary

An evaluation of the concepts for the trace determination of prostaglandins (PGs) by tandem mass spectrometry (MS/MS) has been achieved. Results from the studies in Chapter 3 demonstrate the importance of the optimization of various parameters in the collisionally-activated dissociation (CAD) process before performing trace analysis. Optimization of both collision energy and collision gas pressure is essential in obtaining an accurate qualitative daughter spectrum "rich" in structural information. Choice of a particular daughter ion for SRM is essential and can affect the sensitivity and/or selectivity of an analysis. The CAD reaction with the highest CAD efficiency should be selected to yield the highest sensitivity for SRM determination of PGs. A dramatic difference in the optimum collision gas pressure for SRM with MS/MS for the determination of PGE_2 and $PGF_{2\alpha}$ has been observed and discussed.

The differences in fragmentation behavior of PGE_2 and $PGF_{2\alpha}$ were examined through the use of fragmentation, collection and overall CAD efficiency studies. This work showed that the CAD efficiency for the $[MO/TMS\text{-}PFB]^-$, $[M\text{-}PFB]^-$ and $[M\text{-}H]^-$ carboxylate anions is significantly different for subtle structural changes in PGs. Through the use of these

curves, we have shown that F-series PGs fragment less efficiently than PGE_2 and PGD_2. A possible explanation has been proposed to explain these dramatic differences seen in the fragmentation behavior of structurally similar carboxylate anions of PGs. It is believed that F-series PGs are more stable during CAD due to the interaction of the carboxylate group with the two -OH groups in the cyclopentyl ring.

The advantages and limitations of immunoaffinity purification (IA) for sample preparation of PGs in urine have been investigated. Results show that IA purification coupled with a short 3 m GC capillary column utilizing EC-NCI selected-reaction monitoring (SRM) can provide a selective, sensitive and rapid method of analysis for endogenous levels of PGE_2 in urine. The studies performed in Chapters 2 and 5 demonstrate the reusability of the IA sorbent and its capabilities in various analytical schemes. However, data from Chapter 6 shows that IA purification still has limitations. The non-specific binding which occurs due to secondary interactions in the IA procedure creates difficulties in analysis when less selective sample introduction (direct probe or DCI) or detection schemes (selected-ion monitoring) are employed.

The systematic study which was performed in Chapter 5 demonstrates the relative trade-offs which exist throughout the entire analytical procedure. This study indicates that the utilization of a more selective sample preparation method (IA) with MS/MS can reduce the chromatographic separation time required to achieve the necessary selectivity and sensitivity for PG analysis in urine. However, results show that MS/MS is not necessary if IA purification and a longer chromatographic separation (more selectivity) technique are employed. In addition,

dramatic differences in sensitivity were observed for PGE_2 for the three different lengths of GC columns employed. This type of systematic study should be applicable in the evaluation of any analytical procedure for analysis of components in a biological sample.

Future Directions

Validation of the accuracy and effectiveness of IA purification coupled with a 3 m GC capillary column and SRM needs to be performed on samples which have already been analyzed with another method, such as radioimmunoassay (RIA). Another aspect of IA which requires immediate attention is the applicability to other matrices, especially human milk samples. This will possibly require an optimization of the extraction technique with the use of different solvents. In addition, the sensitivity of this method requires further examination, if it is to be employed for the routine analysis of small prenatal samples.

A number of studies are still needed to establish an optimum method of analysis for PGs in biological samples utilizing MS/MS. Various sample preparations, derivation procedures, ionization and measurement methods need to be evaluated. Even though IA purification is a reliable, selective method for sample preparation, the optimization of an even simpler, selective, more rapid extraction procedure would be highly advantageous. The commercial availability of monoclonal antibodies for PG analysis would reduce sample preparation time even more and possibly reduce the non-specific binding problems observed with the polyclonal immunoafffinity columns tested. This would allow further evaluation of

direct chemical ionization (DCI) as a possible rapid sample introduction technique.

Derivatization procedures for SRM also need to be addressed. The use of EC-NCI GC/MS utilizes the MO/PFB/TMS derivative for analysis. This approach has served as the basis of the more sensitive and selective analysis by SIM (48-52). In this work, it was found that the intense carboxylate anion formed from this derivatization produced inefficient overall CAD (< 2.0%) for F-series PGs. Furthermore, with EC-NCI, derivative-specific fragmentation is prominent with little to no "backbone-specific" fragmentation present. Therefore, further examination of more effective derivatization procedures with various ionization methods for SRM analysis need to be performed.

Along these lines, Strife (90) has performed preliminary studies with the ME/MO/TMS derivative utilizing the ion trap mass spectrometer (ITMS), discussed in Chapter 1. The ITMS offers access to "backbone-specific" fragmentation, but at high CAD efficiencies of 50% (91). This method of measurement has the additional advantage of not sacrificing sensitivity for selectivity when using the MS/MS mode of operation. In initial trials, he has observed excellent sensitivity for less than 75-pg quantities of PGs injected on column.

From these studies and the literature, it is clear that much work remains to be performed in the systematic optimization of the analytical scheme for the analysis of endogenous levels of PGs in biological samples. The principles discussed in this work, coupled with new analytical techniques may help to influence future studies in PG analysis.

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BIOGRAPHICAL SKETCH

Todd Allen Gillespie was born in Portland, Indiana on January 13, 1962. He attended Jay County High School, graduating in 1980 as the senior class president. From 1980 to 1984 while attending the University of Indianapolis, he lettered four years on the collegiate tennis team and was head statistician for the football and basketball teams from 1983 to 1984. During his senior year at U. of I. he acquired an interest in analytical chemistry while employed as a co-op at Merrell Dow Pharmaceutical, Inc. in Indianapolis. He graduated Cum Laude in May 1984 with a B.S. degree in chemistry and mathematics. In August of 1988, he began his graduate school studies at the University of Florida under the direction of Dr. Richard A. Yost. During the spring of 1987, he married his beautiful wife Paula E. Hannon at the University of Indianapolis. Upon graduation his wife and him will be moving to Cincinnati, Ohio where Todd will be employed at Merrell Dow Research Institute.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard A. Yost, Chairman
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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